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ROLE OF PROSTAGLANDIN E2 AS A NEURO-IMMUNE MEDIATOR IN THE CHOLINERGIC ANTI-INFLAMMATORY PATHWAY

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Role of Prostaglandin E2 as a Neuro-Immune Mediator in the Cholinergic Anti-inflammatory Pathway

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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கற்றது கைமண் அளவு, கல்லாதது உலகளவு

- அவ்வையார்

What you have learned is a mere handful;
What you haven't learned is the size of the world

- Avvaiyar

Dedicated to all the animals and donors included in my thesis

ABSTRACT

Therapeutic implications of cholinergic anti-inflammatory pathway (CAP) activation have marked the beginning of a novel treatment strategy of passing electrical impulses along the left vagus nerve to alleviate acute and chronic inflammatory conditions. Extensive work in understanding CAP through vagus nerve stimulation (VNS) have identified key molecular and cellular mechanisms involved in the α7 nicotinic acetylcholine receptor (nAChR) mediated control of inflammation. Interestingly, recent studies have implicated a role of prostaglandin E2 (PGE_2), whose concentration is increased locally following α7 nAChR activation, in this neuro-immune crosstalk. PGE_2 is a potent lipid mediator produced by the action of cyclooxygenase enzymes (COX-1/2) and other specific downstream enzymes such as microsomal prostaglandin synthase-1 (mPGES-1) on the phospholipase-released arachidonic acid. While the role of PGE_2 during inflammation is still debated, it has now gained fresh impetus owing to recent findings on its role in resolving inflammation. In addition, our knowledge concerning the role of PGE_2 in the immunomodulatory effects of CAP is limited. Here, we have investigated the importance of PGE_2 in the cholinergic regulation of inflammation by performing both *in vivo* and *in vitro* studies with mPGES-1 gene deleted (mPGES-1 (-/-)) mice and human astroglial cells subjected to VNS and nicotine treatment respectively. We observed that inducible PGE_2 production is crucial for VNS mediated splenic cholinergic synthesis and immunosuppressive effects during endotoxemia. These observations were applicable only to the splenic portion of the CAP, since VNS-induced norepinephrine release from the splenic nerve was not dependent on PGE_2, nor was the expression of β2-adrenergic receptors (AR) on effector T lymphocytes. Importantly, mPGES-1 pharmacological inhibition reversed nicotine immunomodulatory effects in LPS activated human peripheral blood mononuclear cells. Interestingly, while VNS did not affect the COX-1/2 expression, hypothalamic mPGES-1 was found to be elevated. In addition, COX-2 mediated PGE_2 is involved in the cholinergic suppression of reactive astrogliosis and neuroinflammatory conditions. In conclusion, we propose that PGE_2, which is known to drive febrile response and inflammatory pain during early stages of inflammation, can also have a regulatory role in the cholinergic immune regulation.

LIST OF SCIENTIFIC PAPERS

I. Le Maître, E., **Revathikumar, P**., Idborg, H., Raouf, J., Korotkova, M., Jakobsson, P. J., Lampa, J. **'Impaired vagus-mediated immunosuppression in microsomal prostaglandin E synthase-1 deficient mice'**, *Prostaglandins & other lipid mediators, 2015; 121: 155-62*.

II. **Revathikumar, P**., Karmakar, U., Estelius, J., Le Maître, E., Korotkova, M., Jakobsson, P. J., Lampa, J. **'Microsomal Prostaglandin E synthase-1 gene deletion impairs neuro-immune circuitry of cholinergic anti-inflammatory pathway in endotoxemic mouse spleen'.** Manuscript.

III. **Revathikumar, P**., Le Maître, E., Korotkova, M., Hökfelt, T., Svensson, C., Jakobsson, P. J., Lampa, J. **'Vagus nerve stimulation augments microsomal prostaglandin synthase-1 protein expression and inhibits substance P expression in specific regions of the endotoxemic mice brain '.** Manuscript.

IV. **Revathikumar, P**., Bergqvist, F., Gopalakrishnan, S., Korotkova, M., Jakobsson, P. J., Lampa, J*., Le Maître*, E. **'Immunomodulatory effects of nicotine on interleukin 1β activated human astrocytes and the role of cyclooxygenase 2 in the underlying mechanism'**, *Journal of Neuroinflammation, 2016;13: 1-13*.

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LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THESIS

V.

Le Maître E, **Revathikumar, P**., Estelius, J., Lampa, J. **'Increased Recovery Time and Decreased LPS Administration to Study the Vagus Nerve Stimulation Mechanisms in Limited Inflammatory Responses'**. *Journal of Visualized Experiments*. *2017:121 (epub) doi: 10.3791/54890*

CONTENTS

LIST OF ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetyl cholinesterase
ACTH	Adrenocorticotrophic hormone
Ag	Antigen
ANS	Autonomic nervous system
AR	Adrenergic receptor
BuChE	Butyrylcholinesterase
Ca^{2+}	Calcium ion
CAIA	Collagen antibody induced arthritis
cAMP	Cyclic adenosine monophosphate
CAP	Cholinergic anti-inflammatory pathway
ChAT	Choline acetyltransferase
CIA	Collagen induced arthritis
CNS	Central nervous system
COX-1/2	Cyclooxygenases-1/2
cPGES	Cytosolic prostaglandin E synthase
CRH	Corticotrophin releasing hormone
CXCL1	Chemokine (C-X-C motif) ligand 1
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DMV	Dorsal motor nucleus of the vagus
DRN	Dorsal raphe nucleus
HPA	Hypothalamic-pituitary-adrenal
IFNγ	Interferon γ
IL-10	Interleukin-10
IL-13	Interleukin-13
IL-1β	Interleukin-1beta
IL-6	Interleukin-6
IL-8	Interleukin-8
JAK2	Janus kinase 2

LC	Locus coeruleus
LPS	Lipopolysaccharide
MAPEG	Membrane Associated Proteins in Eicosanoid and Glutathione metabolism
MHC	Major histocompatibility complex
mPGES-1	Microsomal prostaglandin E synthase-1
MS	Multiple sclerosis
Na^+	Sodium ion
NE	Norepinephrine
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSAIDS	Nonsteroidal anti-inflammatory drugs
NTS	Nucleus tractus solitarius
PAG	Periaqueductal gray
PAMP	Pathogen associated molecular pattern
PG	Prostaglandins
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGI_2	Prostaglandin I_2
PHA	Phytohaemagglutinin
PKA	Protein kinase A
PKC	Protein kinase C
PNS	Peripheral nervous system
RA	Rheumatoid arthritis
SEM	Standard error mean
SN	Splenic nerve
SNS	Somatic nervous system
SOCS 3	Suppressor of cytokine signaling 3
SPMs	Special pro resolving mediators
STAT3	Signal Transducer and Activator of Transcription 3
TGFβ	Transforming Growth Factor β

Th1	T helper cell type 1
Th17	T helper cell type 17
Th2	T helper cell type 2
TNFα	Tumor necrosis factor alpha
TXA_2	Thromboxane A_2
VNS	Vagus nerve stimulation
α7nAChR	Alpha 7 nicotinic acetylcholine receptor

1 INTRODUCTION

1.1 INFLAMMATION AND IMMUNITY

The immune system plays a fundamental role in protecting us from life threatening pathogens and toxic substances. Immunity can be broadly classified as innate or adaptive. Innate immunity instantaneously provides the first line of protection against invading pathogens in organisms as small as fungi to highly complex vertebrates. It predominantly identifies pathogens through pathogen (PAMPs) and danger associated (DAMPs) molecular patterns expressed by innate immune cells. The key players involved in innate immunity include dendritic cells, macrophages, monocytes, neutrophils, eosinophils, basophils and mast cells. Interestingly, each one of them deploy a unique combination of defense strategies to destroy pathogens. In addition to phagocytosis, while neutrophils secrete large quantities of cytotoxic reactive oxygen species and proinflammatory cytokines like TNF and IL-12, macrophages and monocytes use nitric oxide to kill microbes. Intriguingly, phenotype (M1/M2) of macrophages engaged in an immune response is largely determined by the signals present during their differentiation and activation. Classically activated macrophages (M1) produce high levels of proinflammatory cytokines such as TNF α, IFN-γ, IL-6 and IL-12 and possess potent antibacterial properties (1). Alternatively, macrophages (M2) generated in the presence of IL-4, IL-10, IL-13 and particularly glucocorticoids, usually secrete anti-inflammatory cytokines and thus play an immunoregulatory function. Immediate hypersensitivity responses against allergens and immune response towards helminths and other parasites are mainly conducted by eosinophils, basophils and mast cells. Upon trigger, these innate immune cells release preformed toxic mediators from their cytoplasmic granules and secrete lipid mediators, thereby initiating tissue inflammation and edema (1).

Apart from primarily initiating the immune response, innate immunity also activates and educates the adaptive immunity to carry out the secondary immune responses in an antigen specific manner. Importantly, antigen presenting cells such as macrophages and dendritic cells (DCs) are known to phagocytose pathogens, break them down into smaller peptide fragments that can be presented by major histocompatibility complex (MHC 1 and II) on their cell surface for antigen recognition by T cells. Depending on the MHC-antigen (Ag) complex presented and other costimulatory signals received, different subsets of T cells can be activated. For instance, $CD8^+$ cytotoxic T cells predominantly kill intracellular pathogens and $CD4^+$ helper T lymphocytes carry out cellular and humoral immune responses (B cell maturation) by engaging MHC I and II-Ag complexes respectively. $CD4^+$ T helper population include different subsets such as Th1, Th2 and Th17 with varying effector functions. While Th1 and Th2 subpopulations are implicated in cell mediated and humoral/allergic immune responses, Th17 cells are responsible for the combat against extracellular bacteria, neutrophil recruitment to clear pathogens and destructive inflammatory response in autoimmunity. In contrast, a specified percentage of circulating helper T cells is established to participate in

down regulating immune responses by producing immunomodulatory cytokines such as IL-10 and TGFβ and are specifically called regulatory T cells (T_{reg}).

Inflammation, irrespective of the noxious stimuli, is coordinated by a surplus of cytokines and chemokines involved in recruiting immune cells to inflamed site, thereby inducing the cardinal signs of inflammation such as redness, swelling, pain, loss of function and fever. While the extent and intensity of inflammation mounted by an individual is determined by several factors, resolution of inflammation and restoration of homeostasis after the threat is eliminated is pivotal to keep onset of chronic inflammatory diseases at bay. Humoral (e.g. glucocorticoids) and cellular mechanisms (regulatory T-cells, M2 macrophages) are extensively studied for their role in regulating immune responses (2). Intriguingly, groundbreaking advances in the field of neuroimmunology have discovered specific neural mechanisms with immense therapeutic potential to control inflammation both in CNS and periphery (3).

1.2 NERVOUS SYSTEM – AN OVERVIEW

Human nervous system can be broadly classified as central (CNS) and peripheral nervous system (PNS). While CNS includes both brain and spinal cord, the PNS can be divided into autonomic (ANS) and somatic nervous system (SNS). Importantly, ANS is known to innervate cardiac muscle, smooth muscle and numerous glands and maintain homeostasis involuntarily. Many of the basic bodily functions are regulated by autonomic reflexes where sensory nerves convey important information from the periphery to respective control centers in CNS, incoming impulses are processed and a corresponding response is sent out through autonomic nerves to restore homeostasis. The efferent pathway of an autonomic reflex consists of two neurons. Preganglionic neurons emerging from CNS ends in an autonomic ganglion located outside CNS and connect with a post ganglionic neuron innervating the target tissue. Such reflexes support and sustain several vital processes namely blood pressure, heart rate, gastrointestinal peristalsis, body temperature etc.

ANS can be further subdivided as either sympathetic (“fight or flight” response) or parasympathetic (“rest and eat” response). Both divisions are anatomically and functionally different with the ability to maintain effector functions in a tonic manner. Interestingly, 75% of the parasympathetic fibers belong to the vagus nerve which innervates several organs situated in the thorax and abdomen. The primary neurotransmitters released by ANS fibers are norepinephrine (NE) (adrenergic) and acetylcholine (ACh) (cholinergic) respectively. While all the preganglionic nerves of the ANS, post ganglionic nerves of parasympathetic system are cholinergic, postganglionic fibers of sympathetic system are mostly adrenergic. Sympathetic and parasympathetic post ganglionic nerves often exert opposing effects on the effector tissue. After exerting their effects, these neurotransmitters are rapidly cleared from their neuroeffector junctions by dedicated enzymatic degradation of ACh into choline and acetate or by reuptake of NE into the sympathetic nerve that released it. These neurotransmitters can either inhibit or enhance the target function depending on the type of

receptor it activates on the cell surface. ACh can bind to two different types of receptors, nicotinic and muscarinic. Cell bodies of all post ganglionic nerves of ANS express nicotinic receptors and upon stimulation increase the intracellular Na^+ and Ca^{2+} ion concentration resulting in excitation of the corresponding nerve fibers. On the other hand, muscarinic receptors are mostly found on the effector tissue and coupled to G proteins and second messengers that carry out intracellular outcomes. In case of NE, the adrenergic receptors can be divided as α and β. Each of these receptor groups can be further subdivided into different categories depending on their functions, intracellular pathways and location. (4)

1.3 NEURAL REGULATING PATHWAYS

1.3.1 Sickness syndrome and HPA axis

Proinflammatory cytokines induced by systemic inflammation can reach the CNS either through circumventricular regions, activation of cytokine receptors or by induction of prostaglandins (PGs) across the blood brain barrier (4). CNS, in turn, orchestrates various symptoms of sickness behavior including fever, pain, reduced food intake and social activity. Brain endothelial and hypothalamic expression of PGE_2 related enzymes such as COX-1/2 and mPGES-1 are shown to play key roles in inducing sickness syndrome and hypothalamic-pituitary-adrenal (HPA) axis, two main neural reflex responses to an immunological challenge (5). Increase in central PGE_2 production at the cerebrovascular lining can diffuse into deep brain structures responsible for neural responses to systemic inflammation. For example, activation of EP3 (PGE_2 receptor) bearing cytokine sensitive neurons in specific regions such as hypothalamus and medial preoptic area are shown to be involved in corticotrophin releasing hormone (CRH) upregulation and induction of febrile and pain responses (6). HPA axis, carried out by EP3 receptor activation on sympathetic neurons in hypothalamic paraventricular nucleus, sends out crucial negative feedback signal to immune system (7, 8). CRH expression in hypothalamus eventually leads to adrenocorticotrophic hormone (ACTH) release and subsequent upregulation of anti-inflammatory mediators, glucocorticoids, in the periphery. Glucocorticoids are well studied to suppress inflammation induced cytokine release and aid in restoring homeostasis in the host.

1.3.2 Sympathetic regulation of immune responses

Noradrenergic sympathetic nerves innervate primary and secondary lymphoid organs where the activated nerve endings can release NE in close proximity to many immune cell types such as macrophages, DCs, lymphocytes and others. Electron microscopic studies have clearly demonstrated the sympathetic nerve terminals in juxtaposition to T lymphocytes located in the splenic white pulp. β_2 AR stimulation at physiological levels have been shown to mitigate acute and chronic inflammation partly by inhibiting important proinflammatory cytokines such as TNF and IL-1, limiting cellular immune response and enhancing Th2 mediated antibody immunological action. The shift towards Th2 activity is achieved by increasing IL-4 and IL-10 cytokine release and at the same time, suppressing IFN-γ and IL-12

levels. On the contrary, stimulation of α ARs increase TNF and IL-1 production and decrease IL-10 and IL-6, thereby fueling inflammation. Adding to the complexity, local NE concentration can have a differential impact on the target with high NE levels being generally inhibitory and low NE exhibiting excitatory effects. Thus, consequence of NE release on local immune population is largely determined by the type and density of adrenergic receptor (AR) expressed on the cell surface, concentration of NE in the milieu, G protein coupled to AR and activation status of the target (9).

Intravenously injected LPS can activate sympathetic splanchnic and splenic nerve activity leading to an increase in plasma NE levels (10, 11). Interestingly, ANS has been associated with autonomic neural reflexes shown to dampen acute inflammation. For example, NE release in response to activation of the splenic nerve can suppress the production of proinflammatory cytokines through direct activation of its β_2 receptors on activated macrophages (12). Furthermore, voluntary activation of sympathetic neural system in humans inhibited the inflammatory response mounted against LPS (13) and severing splanchnic nerve fibers increased LPS induced plasma TNFα levels (14). Increase in plasma TNFα levels following severed splanchnic nerve fibers and failure of vagotomy to abrogate splenic nerve activity in LPS treated animals led to the emergence of splanchnic anti-inflammatory pathway respectively (11). Such anti-inflammatory effects of sympathetic efferents are also demonstrated in zymosan induced air pouch model (15) and abdominal inflammation (16).

1.3.3 Parasympathetic regulation of immune responses

Induction of fever by even very low doses of LPS which do not produce measureable levels of cytokines in the periphery led to the discovery of afferent nerve signaling in immune to brain communication (17). The immune system has the ability to directly communicate with the CNS through cytokine (IL-1β) dependent activation of the afferent vagus nerves, which in turn, activate specific regions in the brainstem such as nucleus tractus solitarius (NTS) and dorsal motor nucleus (DMV) (18). The vagus nerve is the tenth cranial nerve which mainly originates from the cranium and innervates all principal visceral organs in a subconscious manner. The vagus nerve relays information to and from CNS and aids in maintaining behavioral and physiological homeostasis. A majority of the vagal afferents (nearly 80%) terminate in the NTS at the brainstem and known to control gastrointestinal, cardiovascular and respiratory systems (19). In addition, acute inflammatory models involving sub diaphragmatic vagotomy implicated the importance of afferent vagal signaling in mediating sickness syndrome and HPA activation in response to low doses of LPS or cytokines (20, 21). Interestingly, afferent vagus nerve fibers also seem to activate a central processing network leading to the activation of the efferent vagus nerve which effectively down regulates inflammation by inhibiting harmful cytokine production. Such a reflex mechanism solely dependent on the bidirectional neuro-immune communication became popularly known as the ‘inflammatory reflex’ (22). Functional studies on the inflammatory reflex identified ACh as the principal neurotransmitter in limiting inflammation following the efferent vagus nerve

stimulation. Hence, the efferent arm of the inflammatory reflex is called the 'Cholinergic Anti-inflammatory Pathway' (CAP).

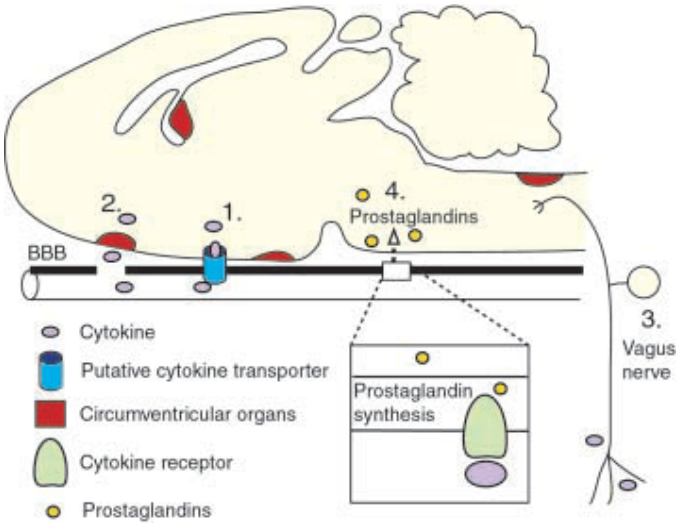

Figure 1. Immune to brain signaling. Our immune system can function as a sense organ to the CNS and convey key signals about the immunological status in multiple ways. Inflammatory cytokines can reach the CNS by 1. Active transport across blood brain barrier (BBB) 2. Passage through the circumventricular regions lacking BBB 3. Afferent vagal signaling and 4. Induction of PGE2 synthesis across the brain endothelial cells.

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1.4 CHOLINERGIC ANTI-INFLAMMATORY PATHWAY

1.4.1 Overview

Tracey and colleagues have performed extensive research on the anatomical details of the CAP and proposed a combined parasympathetic-sympathetic nerve model composed of vagus and splenic nerve respectively (3). Electrical stimulation of the cholinergic efferent vagus nerve ending in the celiac-mesenteric ganglion activates the adrenergic splenic nerve by a mechanism yet to be understood. The splenic nerve, in turn, releases NE in juxtaposition to immune cells in marginal zone, white and red pulp of the spleen, an important organ of CAP (23, 24). NE from the splenic nerve endings binds to its corresponding β_2 AR expressed on effector memory T lymphocytes and induces ACh formation through action of choline acetyl transferase (ChAT) (25). Released ACh from T cells will then activate the α7nAChR on macrophages (26), thereby limiting cytokine production and inflammation as shown in **Figure 2**. In fact, CAP activation by VNS failed to limit inflammation and conversely, increased TNF production following endotoxin treatment in α7nAChR knock out mice (26). However, recent studies have also suggested the possibility of a direct action of NE on macrophages in bringing down inflammation (27). Hence, vagus nerve, splenic nerve and spleen become the main components of CAP to fine tune inflammation in a localized, specific and discrete manner (23, 28, 29). Apart from their profound effects on innate

immunity, CAP activation also modulated T cell independent B cell humoral response to heat activated *Streptococcus pneumoniae* where B cell migration and subsequent antibody production was obstructed (30). Furthermore, treatment with nicotine, a potent α7nAChR agonist, strongly reduced the Th1 and Th17 proliferation and associated cytokine levels and favored Th2 response which proved to be beneficial for alleviating disease severity in case of experimental autoimmune encephalomyelitis (31)

1.4.2 Intracellular events of α7nACh receptor signaling

Acetylcholine receptors are mainly classified as nicotinic and muscarinic receptors associated with different functions. M1 muscarinic receptors are mainly involved in the complex brain signaling of CAP (32). However, the peripheral effects of CAP are primarily carried out by the nicotinic receptors on activated macrophages. They are either homo- or hetero-pentameric ligand gated ion channels composed of 17 different subunits (α 1-10, β1-4, γ, δ, ε). Special attention is given to the α7nACh receptors that are detrimental to the integrity of CAP (33). Neuronal α7nACh receptors are usually homo-pentameric in nature and act as calcium channels involved in the regulation of neurotransmitter release in presynaptic nerve terminals and excitation of postsynaptic nerves. Apart from neurons, glial cells also express α7nACh receptors whose activation by ACh or nicotine can mediate neuroprotective effects during inflammation (34, 35). The intracellular mechanisms following activation of α7nACh receptors are well characterized in CNS (36). While the neuronal α7nACh receptors are associated with neuronal plasticity and survival (37), in contrast, their peripheral counterparts are involved in controlling inflammation and thus linked to a different intracellular pathway. Anti-inflammatory effects of α7nAChR are attributed to NF-kB inhibition and activation of JAK2/STAT3 signaling (38, 39). α7nACh receptor activation inhibits NF-κB migration to the nucleus by increasing IκB expression in human endothelial cells whereas the same outcome is achieved by inhibiting IκB phosphorylation and subsequent degradation in human monocytes (40, 41). Thus, different mechanisms are proposed depending on the cell type. On the other hand, α7 activation can also enhance the active STAT3 concentration intracellularly, which can either directly bind to NFκB and block its interaction with the DNA (42) or induce suppressor of cytokine signaling 3 (SOCS 3) expression (43). Altogether, the convergence of different molecular mechanisms in limiting cytokine release and possibility of other signaling pathways triggered by α7 ligation needs to be addressed in detail.

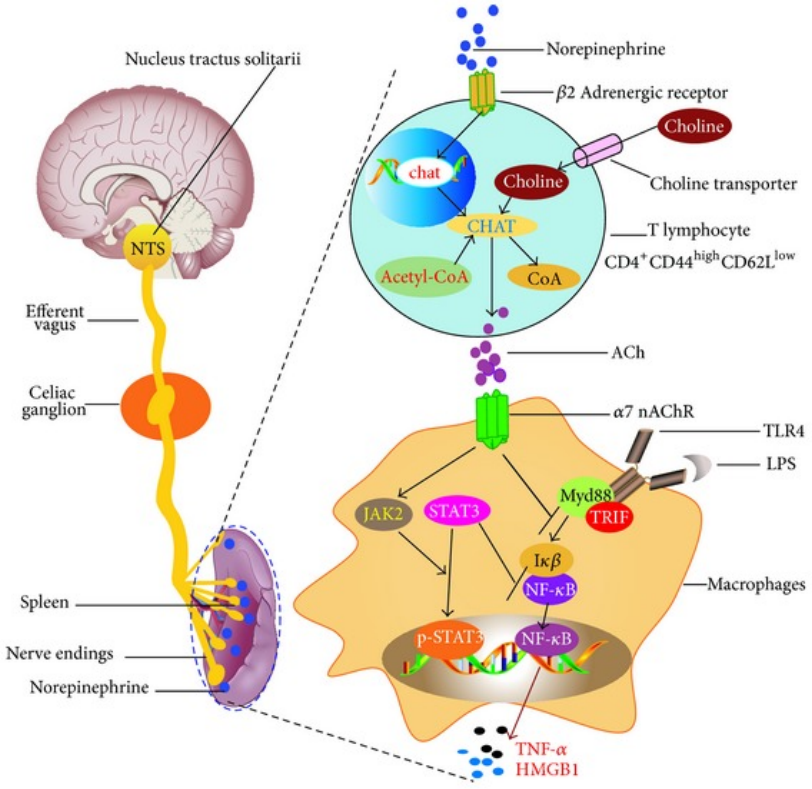

Figure 2. The Cholinergic Anti-inflammatory Pathway. Action potentials along the efferent vagus nerve induce NE release from splenic nerve endings. β_2 AR activation results in increased acetylcholine production by effector memory cells ($CD44^{hi}$ $CD62L^{lo}$) which in turn mediates immunosuppressive effects on splenic macrophages via α7nAChRs.

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1.4.3 Central projections of the vagus nerve

Afferent vagal nerve fibers mainly project bilaterally to NTS in the medulla oblongata from where it directly enters parabrachial nuclei, the cerebellum, the raphe, the periaqueductal gray (PAG) and the locus coeruleus (LC) (45). Through the NTS, the vagus nerve also makes projections to the central cholinergic network composed of cortex, paraventricular nucleus, and lateral hypothalamic region among others. Activation of this network sends output signals through periaqueductal gray (midbrain), parabrachial nucleus (pons) and NTS, nucleus ambiguus and ventrolateral medulla (medullary nuclei) and conveyed to the periphery by the efferent vagus nerve (46).

Stimulation of the vagus nerve results in NE production in the LC, primary source of NE in the brain. Interestingly, there are direct adrenergic nerve projections from LC to forebrain basal cholinergic system (47, 48) and NE release from LC can thus activate the brain cholinergic neurons via α_1 receptors (49). Similar to the effects of CAP in the periphery, ACh has been shown to have neuroprotective effects on the activated microglia mediated by α7nAChR (34, 50) as illustrated in **Figure 3**. In parallel, selective M1 muscarinic receptor agonists have been elucidated to have positive effects on Alzheimer and schizophrenia (51).

In addition, projections from LC result in serotonin release at the dorsal raphe nucleus (DRN) that in turn can inhibit excitotoxicity by inhibiting glutamate release (52) and reducing NR2B subunit of N-methyl-D-aspartate receptor (53). Thus, CAP activation through electrical vagus nerve stimulation has the potential to also activate a cholinergic pathway in the brain and thereby control neuroinflammation.

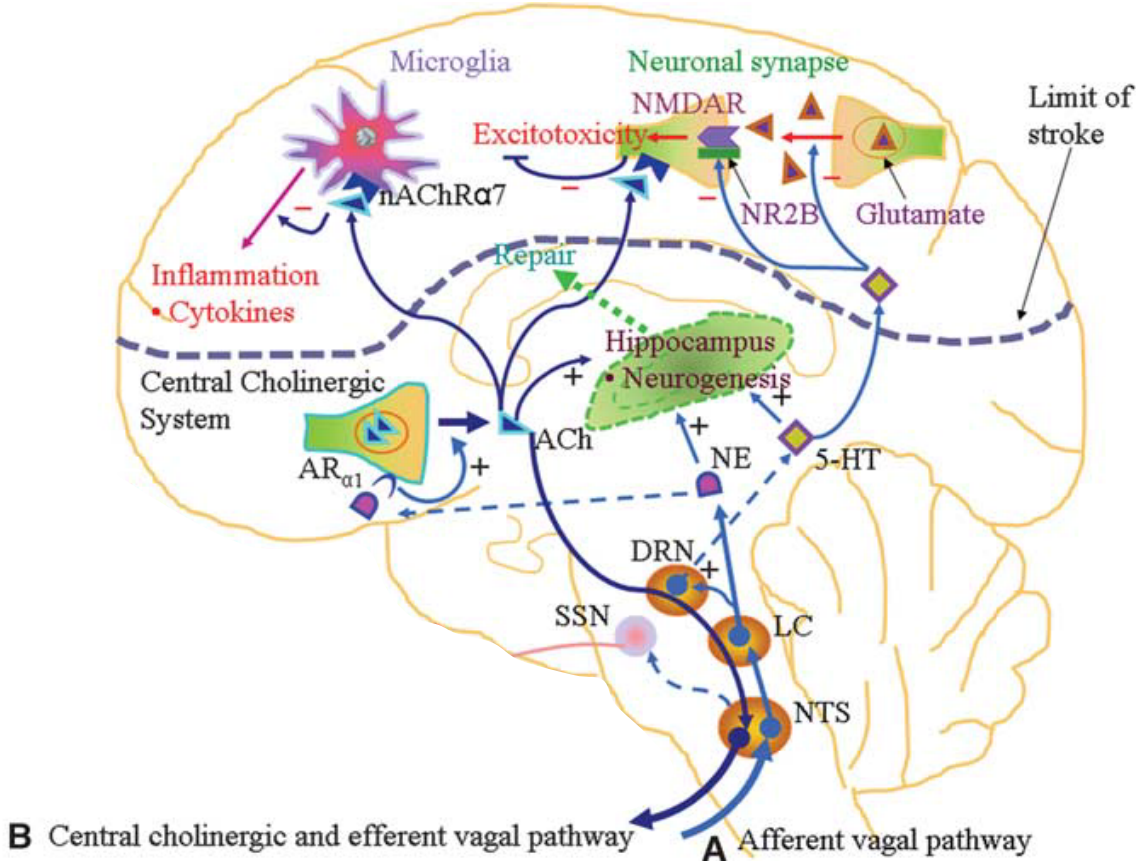

Figure 3. Potential neuroprotective effects of CAP. The afferent vagal pathway is represented by deep blue arrows and the efferent pathway by dark blue arrows. Majority of the vagal afferents converge at the NTS which upon activation induce NE release from the LC. NE subsequently increases the serotonin (5-hydoxytryptamine, 5-HT) levels at DRN. NE activates the central cholinergic network through α_1AR resulting in ACh production and thereby limiting neuroinflammation through α7nAChR. On the other hand, 5-HT combats excitotoxicity by inhibiting glutamate release and NR2B expression. Apart from releasing ACh, central cholinergic network also sends anti-inflammatory signals through efferent vagus nerve fibers to the periphery.

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1.4.4 Acetylcholine and inflammation

Acetylcholine is synthesized by the enzymatic action of ChAT on the precursors choline and acetyl coenzyme A (acetyl-coA) respectively in the cytoplasm of preganglionic cholinergic nerve terminals. ACh is then transported into vesicles by specialized vesicular transporter system (VAChT)and upon stimulation, ACh is actively exocytosed into the synapses

resulting in the activation of postganglionic nerves. Interestingly, ACh thus synthesized is quickly hydrolyzed into choline and acetate by acetyl cholinesterase (AChE) and butyrylcholinesterase (BuChE) present in the synaptic cleft and circulation. For years, neurons were believed to be the only source of ACh, however, recent studies have challenged this paradigm by demonstrating the intrinsic ability of immune cells, glial cells, endothelial cells and many others to secrete ACh when challenged with endotoxin (55). In the CNS, microglia and astrocytes express ChAT activity and produce ACh in the resting as well as activated state(56). In CAP, splenic T lymphocytes are identified as the main source of ACh and function as a crucial mediator between the neural and immune system. Roses-Ballina (25), have discovered a subset of $CD4^+$ T cells to actively produce ACh in a ChAT dependent manner upon β_2 adrenergic receptor stimulation and thereby activate nicotinic or muscarinic receptors in an autocrine or paracrine fashion. Though CD8+ T cells, dendritic cells, macrophages, mast cells and granulocytes have all been shown to express ChAT, a major portion of ACh found in the circulation comes from $CD4^+$ T cells (57). Furthermore, studies on human leukemia T cell lines displayed the up regulation of ChAT mRNA and protein expression owing to cell-cell adhesion with B lymphocytes, antigen presenting cells and endothelial cells (55). Cholinergic system in such non-neuronal cells appears to be regulated by protein kinase A (PKA), PKC and intracellular calcium concentration (58, 59). Unable to detect the presence of VAChT+ storage vesicles in human lymphocytes Fuji et al., suggested that, unlike the neurons, ACh could be synthesized and released instantaneously by lymphocytes (60). However, the possibility of unidentified storage vesicles cannot be excluded in light of mediatophore, a part of vacuolar type H^+ ATPases (V-ATPase), discovered as an important mediator of ACh release in T cells (61). Thus, the mechanism of ACh release in lymphocytes still remains largely elusive.

1.4.5 Cholinergic agonists and immune regulation

Both T and B cells are known to express muscarinic ($M_1$to M_5) and nicotinic receptors (55). Studies on M_1/ M_5 mAChR knockout mice challenged with ovalbumin are shown to exhibit an impaired IgG_1 production compared to the wild type (WT). In agreement with this finding, splenic mononuclear leukocytes from these KO mice secreted low levels of proinflammatory cytokines compared to WT upon stimulation (62). It is thus tempting to speculate the modulatory role of M_1 and / or M_5 mAChR in regulating the cytokine and antibody production by lymphocytes. In addition, M_3 mAChR seems to play a detrimental role in $CD4^+$ T cell development against parasites (63). However, in the case of CAP, peripheral muscarinic receptors seem to be a mute spectator with muscarinic antagonists unable to inhibit the anti-inflammatory effects of VNS in mice (32). On the other hand, α7nAChRs function as a critical molecular link in CAP and ability of their agonists to limit inflammation as effectively as CAP has opened a pandora box. In recent past, several studies are conducted to decipher their therapeutic effects in chronic inflammatory diseases such as Alzheimer, rheumatoid arthritis, sepsis, stroke etc. (54, 64). For instance, use of nicotine and α7nAChR agonist AR-R17779 ameliorated the severity of collagen-induced arthritis and decreased synovial

inflammation in murine joints. AR-R17779 was more potent in delaying the onset of the disease and protecting the joints from destruction (65). Macrophages can be polarized to M1 or M2 phenotype depending on their microenvironment. In general, M1 are believed to be proinflammatory and M2 to be anti-inflammatory in their function (66). While α7nAChR activation has been shown to protect M2 macrophages from ER stress induced apoptosis(67), it suppresses M1 macrophages infiltration in the adipose tissue, thereby reducing obesity induced inflammation and enhanced insulin sensitivity (68). However, little is known of the potential role of M1/M2 macrophage balance in central lymphatic organs, such as the spleen after activation of CAP. CAP can be activated by electrically stimulating vagus nerve and Tracey and his colleagues have invented a specialized device that can be surgically implanted on the left side of the neck in patients. Trials are currently underway in rheumatoid arthritis (RA) patients to test the clinical significance of VNS in treating chronic inflammation, and the initial results are promising (64). In addition, an alternative strategy could be the use of highly selective cholinergic α7 agonists for activation of CAP and regulation of peripheral inflammation.

1.5 PROSTAGLANDIN E2

1.5.1 Synthesis

Prostaglandins are lipid mediators derived from arachidonic acid, mobilized by phospholipase A_2 enzyme from membrane phospholipids, in response to physiological or pathological stimuli. Arachidonic acid is then converted to prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2) by the enzymatic action of cyclooxygenases. PGH_2 is then rapidly converted into members of the eicosanoid family such as PGE_2, PGD_2, PGI_2, PGF2α or thromboxane (TXA_2) by the terminal synthases as depicted in **Figure 4**.

1.5.2 Cyclooxygenases and their complex role in inflammation

Cyclooxygenases can be classified into COX-1 and 2 respectively. COX-1 is constitutively expressed whereas COX-2 is inducible and strongly associated with inflammation. However, reports have recorded exceptions implicating constitutive COX-2 and inducible COX-1 expression in brain (6). Prostaglandins are produced by a wide range of cells and influence others in paracrine or autocrine fashion through their receptors (EP1-4, IP, TP, DP1-2, FP). A key question in earlier investigations was how peripheral inflammation could cause sickness response, characterized by fever, anorexia and hyperalgesia. Failure to induce fever in COX-2 knock-out mice (69) and induction of fever in response to intracerebroventricular prostaglandin injection (70), shifted the focus towards cerebrovascular COX-2 induced PGE_2 as a key mediator in the neuro-immune crosstalk. This groundbreaking discovery along with studies confirming the pathological role of PGE_2 at inflammatory sites (71) marked the beginning of use of nonsteroidal anti-inflammatory drugs (NSAIDS) and COX-2 inhibitors (NS-398) to treat inflammation and fever.

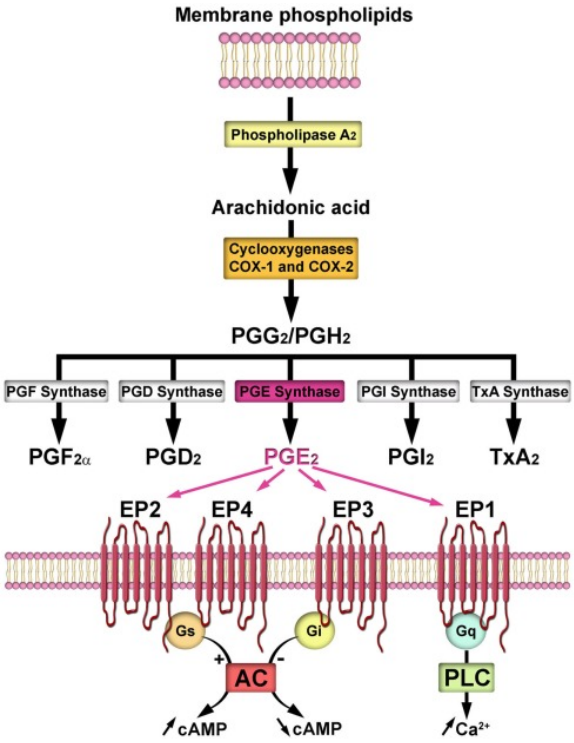

Figure 4. Prostanoid pathway. Arachidonic acid released from the plasma membrane by action of phospholipase A2 is readily converted to endoperoxide intermediates PGG_2 and PGH_2 by cyclooxygenases 1 and 2. Highly unstable intermediates are utilized by terminal synthases such as PGFS, PGDS, PGES, PGIS and TXAS to produce $PGF_{2\alpha}$, PGD_2, PGE_2, PGI_2 and thromboxane A_2 respectively. After its release, PGE_2 exerts its effects by binding to either of its four seven transmembrane G protein coupled receptors ($EP_{1\text{-}4}$). While activation of $EP_{2/4}$ leads to increase in adenylyl cyclase activity and cAMP production, EP_3 bound to inhibitory G protein executes opposite effects. In contrast, EP_1 stimulation enhances intracellular calcium levels through the Gq-phospholipase C pathway.

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NSAIDS include a heterogeneous group of COX-1/2 inhibitors shown extensively to control pain (anti-analgesic), inflammation (anti-inflammatory) and fever (anti-pyretic) in acute inflammatory conditions both in humans (73) and animals (74). However, effects of NSAIDS on chronic inflammation remains largely unknown. In animal RA models, effectiveness of NSAIDS were only restricted to reducing the joint pain and stiffness and did not hinder joint swelling and acute phase protein levels in blood (75). Interestingly, despite strong association with inflammation, COX inhibitors failed to stop the leukocyte infiltration in the synovial tissue and in contrast, induced the production of IL-6 and TNF α (76, 77). Importantly, studies have unraveled the unfavorable effects of chronic use of NSAIDS on cartilage erosion (78), skin (79), bone and tendon wound healing (80, 81). Use of NSAIDS are also linked to deadly side effects on gastrointestinal tract, kidney and cardiovascular system (82) resulting in 103,000 admissions and even worse, 3500 to 16500 deaths per year in the United States (83).

Owing to safety concerns, several NSAIDS such as rofecoxib, valdecoxib and others have been withdrawn from market and efforts are already underway to find alternative targets that can selectively deliver the therapeutic effects of NSAIDS without causing adverse effects on other physiological systems. Further studies in this line of research led to the discovery of the glutathione-dependent enzyme of the MAPEG family, microsomal prostaglandin E synthase (mPGES) as the terminal enzyme functionally coupled to COX-2 and responsible for the up-regulation of central PGE_2 in response to IL-1β in endothelial cells lining the cerebral vessels (84).

1.5.3 Microsomal prostaglandin E synthase-1

mPGES-1, an inducible isomerase of the terminal PGE synthase, is strongly associated with inflammation and pain among other synthases such as cytosolic PGES (cPGES) and mPGES-2 that elicit physiological functions. In fact, cPGES (85) and mPGES-2(86) gene deletion did not result in any significant changes in the PGE_2 levels in animals. Though constitutively expressed in the urinary and reproductive systems, gastric mucosa, spleen, Kupffer cells and resident peritoneal macrophages, it is upregulated several fold in various organs and cell types such as brain, synovial fibroblasts etc. by noxious stimuli such as LPS, IL-1β etc (84, 87). In addition, while cPGES is usually coupled to constitutive COX-1 activity, mPGES-1 is mostly believed to act downstream of COX-2, especially in inflammatory conditions (84, 88). In fact, massive outburst of PGE_2 production seen 6 hours after the intraperitoneal LPS administration was completely abolished to basal levels in mPGES-1 knockout animals implicating its key role in inflammation induced PGE_2 (89).

Genetic deletion of mPGES-1 in mice has confirmed its participation in the pathogenesis of inflammatory diseases such as collagen induced arthritis (CIA), atherosclerosis, LPS induced pyresis, pain, experimental autoimmune encephalomyelitis, Alzheimer, stroke, multiple sclerosis (MS) and cancer (88). Interesingly, Trebino et al, have demonstrated reduced pain and edema formation in acute inflammatory models of mPGES-1 (-/-) mice(90). In chronic inflammatory models such as CIA, mPGES-1 depletion alleviated disease severity and joint damage compared to mPGES-1(+/+) (90). Furthermore, the reduced humoral response was attributed to the mPGES-1 deficiency in mesenchymal stromal cells (91). Clinical studies have shown both Alzheimer and MS patients to have increased COX-2 and mPGES-1 expression in the brain compared to the controls. Neurons, astrocytes, microglia and endothelial cells are the primary source of mPGES-1 in Alzheimer (92). On the contrary, mPGES-1 expression was confined to microglia in MS (93).

More research on understanding mPGES-1 structural properties and its role in inflammation, pain and cancer led to a quest searching for a selective mPGES-1 inhibitor to overcome the shortcomings and adverse effects of traditional NSAIDS and selective COX-2 inhibitors. So far, finding an ideal mPGES-1 inhibitor has been a herculean task owing to interspecies differences, ineffective potency and possible side effects such as impaired healing following myocardial infarction (88). Though deletion of mPGES-1 led to suppression of CIA,

contradictory observations were made in the case of collagen antibody induced arthritis (CAIA) with exacerbated disease severity owing to increased neutrophil recruitment to the inflamed joints (94). Moreover, parallel studies on mPGES-1 gene deleted mice in a chronic neuroinflammatory model displayed an impaired negative feedback mechanism on LPS-induced cytokine synthesis, which was otherwise driven by mPGES-1 derived PGE_2 and EP2 activation in spinal microglia (95). A recent study in mice has shed light on the constitutive mPGES-1 expression in different regions of the brain expressed by different cell types such as pericytes, astroglial cells, leptomeninges and choroid plexus (96) As a consequence, while the pharmacological inhibition of mPGES-1 might be beneficial in the initial phase of inflammation, the possible deleterious effect on resolution cannot be ignored.

1.5.4 Importance of PGE_2 in resolving inflammation

Induction, inflammation and resolution are three distinct stages of an acute inflammatory response. Similar to inflammation, resolution is indeed an active process shown to be driven by pro resolving mediators (97). Interestingly, COX-2 induced PGE_2 is upregulated during the resolution phase of murine arthritis (98). In corroboration, blocking COX-2 activity and subsequent PGE_2 synthesis during the inflammatory phase not only attenuated the magnitude of inflammatory response, but also delayed the resolution and tissue repair (99). These findings thus support use of NSAIDS during the induction phase, but strongly argue against their consumption at later stages of inflammation with potential detrimental effects on resolution and healing. Intriguingly, Chang et al suggest that mPGES-1, owing to its basal gene expression during resolution phase, might not contribute to COX-2/PGE_2 mediated resolution (98). However, role of elevated mPGES-1 activity preceding resolution needs to be investigated.

On the other hand, PGE_2 have also shown to downregulate release of matrix metalloproteinases (MMP -1) by activated synovial fibroblasts, believed to cause tissue destruction in arthritic models (100). Non-selective COX inhibitors resulted in obvious

increase in MMP-1 production. The anti-inflammatory effects mediated by PGE_2 are attributed to ERK and NF-κB inhibition (101). Similar to nicotine, PGE_2 blocks NF-κB activity either by inhibiting the nuclear translocation of p65 subunit or by increasing the IκB expression (102). In reality, several factors in the milieu could determine the effect of PGE_2 during inflammation. For example, in gastric epithelial cells, while PGE_2 inhibited MMP-1 in presence of inflammatory cytokines, their absence reversed its effects (103). Thus, the role played by PGE_2 during inflammation is solely dependent on time, context, concentration and cell type.

1.6 CHOLINERGIC IMMUNE REGULATION AND PROSTAGLANDIN E2

Clinical significance of stimulating CAP is not only limited to treating acute inflammatory conditions, but can potentially be extended to limit chronic inflammation. Accumulating evidence showing its beneficial effects in arresting proinflammatory cytokine release and prolonging survival has promoted its use in treating patients suffering from rheumatoid arthritis (RA), hemorrhagic shock, sepsis, ischemia and other conditions (104). In light of recent findings demonstrating the ability of $CD4^{+}ChAT^{+}$ T cells to regulate blood pressure by stimulating vasorelaxant nitric oxide release(105), the therapeutic capability of VNS has reached new heights. In addition to inflammation, VNS is a well-tolerated treatment for epilepsy and depression and is not known to cause any immunodeficiency and adverse side effects (106). However, it is important to keep in mind that CAP is still not completely understood and existence of possible regulating systems cannot be excluded.

Detailed mechanistic and functional studies on immunomodulatory effects of potent cholinergic agonists such as nicotine on immune cells have contributed immensely to our current understanding of neuro-immune regulation. Nicotine through its α7nAChR ligation blocked the expression of adhesion molecules including ICAM-1, B7.2 and CD40 and proinflammatory cytokines such as IL-12, IFNγ and TNFα in IL-18 stimulated human monocytes and peripheral blood mononuclear cells (PBMC) respectively. Interestingly, these beneficial effects of nicotine were mediated by COX-2 dependent PGE_2 production and corresponding EP2/4 receptors resulting in the elevation of intracellular cAMP secondary messenger concentration and PKA activity (107). Similar effects of nicotine on proinflammatory cytokines via COX 2-PGE_2 pathway have also been studied in immunocompetent glial cells such as microglia (108). Intriguingly, PGE_2 promoted non-small cell lung cancer (NSCLC) cell proliferation through α7nAChR expression following EP_4 mediated activation of c-Jun-N-terminal kinase (JNK), phosphatidylinositol 3-kinase (PI3-K) and PKA pathway(109). Convincing results on activated human leukemic T cell lines (MOLT-3) revealed the crucial role of PGE_2 and its receptor EP_4 in regulating lymphocytic cholinergic activity (110). Thus, prostaglandins seem to be important mediator in the cholinergic signaling and immune regulation.

While the exact role of PGE_2 during inflammation and resolution remains complex, its participation in mediating neuroregulatory events following systemic infection or inflammation is well known. Induction of PGE_2 across the endothelial cells and parenchyma of the brain and subsequent onset of sickness syndrome and activation of HPA axis in response to systemic inflammation are extensively studied (5). In fact, administration of PGE_2 in the CNS resulted in the neuronal stimulation of specific brain structures including NTS, a converging point for afferent vagus nerves (111). Interestingly, presence of IL-1β in the periphery activates the EP3, a subtype of PGE_2 receptor, expressing cells at the vagal nuclei of CNS (18) and more studies with EP3 agonists and KO mice have confirmed EP3 to execute the functional effects of PGE_2 in developing sickness behavior (112, 113). Cytokine

induced action potentials along the afferent vagus nerve is pivotal for HPA activation (20). Thus, PGE_2, apart from mediating immune responses, also serves as a crucial mediator in conveying important signals between the immune and CNS, ultimately resulting in the regulation of immune responses.

Altogether, numerous studies have shown a functional relationship of PGE_2 with cholinergic signaling, and interference with prostaglandin formation or metabolism may have important regulatory effects in the CAP. Moreover, the importance of PGE_2 in initiating resolution may also involve secondary cholinergic immunosuppressive mechanisms and warrants further investigation.

2 AIMS

2.2 GENERAL AIMS

To comprehend the role of prostaglandin E2 as a neuro-immune mediator in the cholinergic anti-inflammatory pathway

2.3 SPECIFIC AIMS

2.3.1 To investigate the significance of mPGES-1 dependent PGE_2 in the immunosuppressive effects of vagus nerve stimulation (VNS) in mice subjected to endotoxemia *(in vivo)*

2.3.2 To dissect the effects of mPGES-1 gene deletion on the neuro-immune circuitry of CAP in endotoxemic mouse spleen (*in vivo* & *in vitro*)

2.3.3 To explore the impact of VNS on the brain PGE_2 related enzymes and substance P neuropeptide expression in endotoxemic mice (*in vivo*)

2.3.4 To study the effects of nicotine on IL-1β activated human astrocytes and role of cyclooxygenase 2 dependent PGE_2 in the cholinergic immune modulation. (*in vitro*)

3 METHODS

3.1 STUDY I

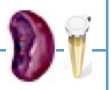
To investigate the significance of mPGES-1 dependent PGE_2 in the immunosuppressive effects of VNS in mice subjected to endotoxemia (*in vivo*)

Animals: In bred DBA/1lacJ mice with Ptges gene deletion (mPGES-1 -/-) and congenic wild type controls (mPGES-1 +/+) were used in our experiments.

Treatment: Isoflurane anesthetized mice underwent an incision at the neck and an electrode was placed under the carefully isolated vagus nerve. The electrode was in turn connected to AcqKnowledge software controlled electric stimulator. Lipopolysaccharide (2mg/kg) was injected intraperitoneally and five minutes later the vagus nerve was stimulated (5V, 1Hz for 5min). After VNS, the wound was stitched and animals were allowed to recover. SHAM animals were only subjected to LPS treatment and superficial neck incision.

Extraction of tissues: Animals were euthanized 6 hours (cytokine/PG profiling) or 30 minutes (ACh measurement) after surgery. Serum from blood, spleen and brain were snap frozen at -80°C.

Cytokine profiling



MesoScale multiplex

Acetylcholine measurement

Choline/ACh assay

3.2 STUDY II

To dissect the effects of mPGES-1 gene deletion on the neuro-immune circuitry of CAP in endotoxemic mouse spleen (*in vivo* & *in vitro*)

Animals: In bred DBA/1lacJ mice with Ptges gene deletion (mPGES-1 -/-) and congenic wild type controls (mPGES-1 +/+) were used in our experiments.

In vivo

Surgery: LPS (2mg/kg) was injected intraperitoneally. Animals underwent either VNS or SHAM

Extraction of tissues: Animals were euthanized 30 minutes after surgery and spleens were collected

Norepinephrine measurement

Competitive ELISA

β_2 adrenergic receptor

Flow Cytometry

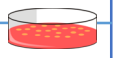
In vitro

Primary splenocytes: Naïve mPGES-1 (+/+) and (-/-) animals were euthanized and spleens were extracted

Treatment: Cells were treated with NE or nicotine 30 min prior to LPS treatment or LPS alone for 3, 6 or 20 h

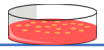
Cytokine profiling

MesoScale multiplex



Choline acetyl transferase expression

Indirect immunofluorescence

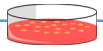


Human PBMC: Fresh PBMC was isolated from buffy coats obtained from healthy donors and cultured in 96 well plates under sterile conditions

Treatment: PBMCs were pretreated with mPGES-1 inhibitor and 30 minutes later stimulated with LPS or LPS+ nicotine simultaneously for 6, 14 and 20 hours

Cytokine measurement

Sandwich ELISA



3.3 STUDY III

To explore the impact of VNS on the brain PGE_2 related enzymes and substance P neuropeptide expression in endotoxemic mice (*in vivo*)

Animals: C57BL/6 mice purchased from Nova-SCB, Sweden were used in our experiments.

Surgery: LPS (2mg/kg) was injected intraperitoneally. Animals underwent either VNS or SHAM

Extraction of tissues: Animals were euthanized 6h after surgery and brains were collected and snap frozen. Coronal sections of the tissue were prepared.

c FOS, COX 1/2 mPGES-1 and SP

In situ hybridization & Immunohistochemistry (ISH & IHC)

mPGES-1 & GFAP

Double immunofluorescence (IF)

Treatment: Intrathecal or intraperitoneal injection of COX inhibitor SC 560 (10 or 100 µg) 30 minutes prior to LPS (2mg/kg) injection

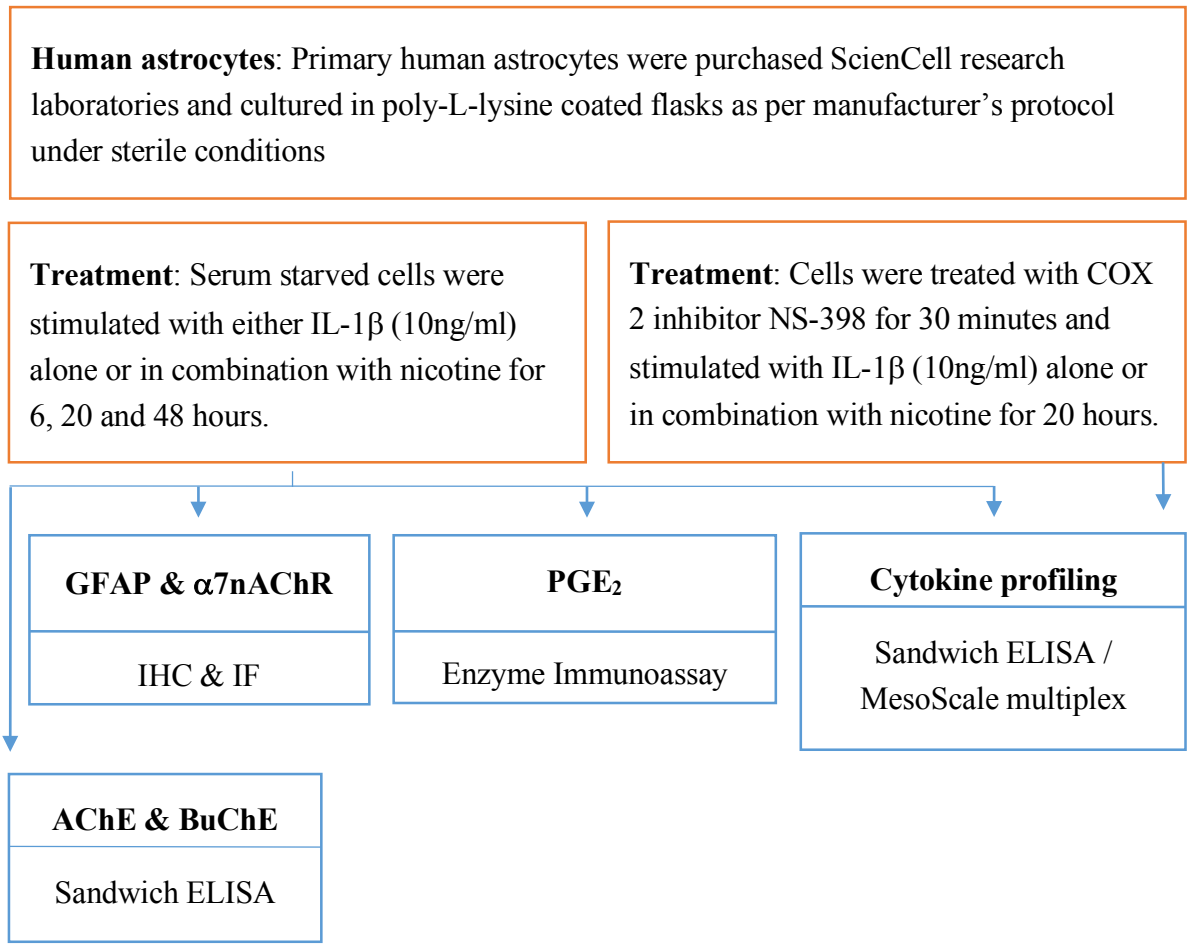
Extraction of tissues: Animals were euthanized 2h or 6h after surgery and brains were collected and homogenized

Cytokine profiling

MesoScale multiplex

3.4 STUDY IV

To study the effects of nicotine on IL-1β activated human astrocytes and role of cyclooxygenase 2 dependent PGE_2 in the cholinergic immune modulation (*in vitro*)



3.5 METHODOLOGICAL CONSIDERATIONS

3.5.1 Extraction of tissue samples

Frozen or fresh tissue samples were homogenized using either polytron PT 10-35 GT or dounce homogenizer on ice. The protease and cyclooxygenase activity in the homogenates was inhibited using Sigma protease inhibitor tablet and indomethacin respectively. Homogenates were transferred to eppendorf tubes and centrifuged at 5000 rpm for 10 min. Supernatants were frozen at -80°C for further analysis for cytokine or PG profiling. This method of sample preparation is well established and used routinely in our experiments.

3.5.2 Measurement of cytokines

Sandwich Enzyme Linked Immuno Sorbent Assay (ELISA)

We measured the mouse TNFα (**Study II**) and human TNFα, IL-6 and IL-8 levels in cell culture supernatants (**Study IV**) using sandwich ELISA. The detection range for mouse TNFα, human TNFα, IL-6 and IL-8 were 31.20 – 2000, 15.60 – 1000, 9.38 - 600 and 31.20 – 2000 pg/mL respectively. Briefly, microtitre plates were first coated with primary antibodies for the respective cytokine overnight and incubated with the samples for 2 hours at room temperature. Following that, biotinylated secondary antibodies were added to the wells and later conjugated to streptavidin-horse radish peroxidase (HRP) conjugate. Finally, substrate solution containing hydrogen peroxide and tetramethylbenzidine (1:1 ratio) was added to individual wells and the color change was measured at 450nm and 562nm was used as the reference wavelength. The standard curve was made using a 4-parameter logistic fit and the concentration of each sample was calculated accordingly. The DuoSet ELISA method offers good sensitivity and specificity. Both fresh and frozen samples can be directly used. The method has no cross reactivity and multiple samples can be assayed in the same plate.

MesoScale multiplex assay

We used mouse (**Study I, II and III**) and human (**Study IV**) 10-plex assay for cytokine profiling of culture supernatants and tissue homogenates. This is an ultrasensitive multiplex kit where each well in the MSD 96-well plate contained 10 carbon electrodes. Each electrode was pre-coated with antibodies against a specific cytokine. Briefly, the wells were first blocked with assay diluent (25 µl), sealed and incubated for 30 min and later incubated with the samples overnight at 4°C with shaking. At the end of the incubation, the detection antibody was added, sealed and incubated for 2h at room temperature. Later, 150 µl of the MSD Read Buffer was added to each well. The MSD plate was read using the MSD Sector Imager 2400 plate reader. The raw data was measured as electrochemiluminescence signal (light) detected by photo detectors and analyzed using the Discovery Workbench 3.0 software (MSD). A 4-parameter logistic fit curve was generated for each analyte using the standards and the concentration of each sample was estimated. The MSD assay has a better sensitivity than cytometric bead array technique. The assay requires small sample volume, less hands-on

time, no prior expertise and offers a wide detection range for a cytokine of interest. However, use of this technique demands a dedicated electrochemiluminescence reader.

3.5.3 Choline/Acetylcholine assay

We homogenized the spleen in the assay buffer and used it immediately to measure the choline and acetylcholine levels by using a simple and sensitive colorimetric method (**Study I**). Free choline is converted to betaine which reacts with the choline probe to develop color at 570nm. The same technique can be used to quantify the total choline content by adding acetylcholinesterase to the reaction mix. Thus, ACh concentration in the supernatant can be calculated by using the formula (total choline – free choline). The assay can detect ACh levels ranging from 10pmol to 5nmol. The method is quick, easy to perform and does not require any special expertise. On the other hand, the method is unsuitable for analyzing already frozen samples. In Study I, we used this assay only to measure ACh levels in freshly isolated spleens. Our goal to quantify ACh levels in NE treated splenocyte culture supernatants (Study II) turned out to be unsuccessful due to the poor sensitivity of the Choline/Acetylcholine kit and lack of time and expertise in optimizing conditions for ACh measurement by LC-MS/MS.

3.5.4 Norepinephrine assay

We quantified the NE levels in the homogenized spleens using competitive ELISA (**Study II**) technique. In short, NE was derived enzymatically and transferred to precoated noradrenaline microtiter plates. The analyte competes for specific number of antibody binding sites and later quantified using anti-rabbit IgG peroxidase conjugate-TMB detection method. The absorbance was read at 450 nm and 630 nm was used as reference wavelength. The sensitivity of the kit is 0.1ng/ml * correction factor and cross reactivity is less than 0.48 %. The detection range is 1.3 – 32000 pg/ml. The disadvantage of this technique is homogenizing the samples in a buffer containing EDTA (1mM) and sodium metabisulfite (4 mM) for avoiding NE degradation makes it unsuitable to be used for measuring other analytes. Similar to ACh, NE can also be effectively measured using LC-MS/MS.

3.5.5 Flow cytometry

β2 adrenergic receptor expression on $CD4^+$ T lymphocytes was measured using flow cytometry (**Study II**). Single cell suspensions of fresh spleens were prepared and $CD4^+$ cells were positively selected using magnetic microbead separation. CD4 enriched cell population was incubated with the following panel of anti-mouse antibodies: PE ADBR2 (bs-0947R-PE, Bioss Antibodies, USA), PE/Cy7 CD 62L (560516, BD Pharmingen, Europe), BV 510 CD 4 (100553, BioLegend, USA), APC Cy7 CD3 (100222, BioLegend, USA) and APC CD44 (103012, BioLegend, USA). Viability of cells was measures by using LIVE/DEAD™ Fixable Green Dead Cell Stain Kit. Stained cells were fixed (4% paraformaldehyde) and samples were acquired the following day using a BD FACSVerse instrument evaluating a minimum of 60000 live single cells. Calculation of compensation matrix and analyzation of

data was performed using FlowJo v.X software. Due to limited number of cells, the compensation was set by using single stained beads and gates were corrected using appropriate FMOs. β2 adrenergic receptor expression was reported as % parent population and mean fluorescence intensity. Flow cytometry technique helps to analyze the expression of the desired protein among specific cell populations and provides a platform to study multiple protein targets. At the same time, the limited choice of antibodies and fluorochromes, non-specific binding and compensation might be considered as limitations.

3.5.6 *In situ* hybridization

This method was used to study c-Fos, COX-2 and substance P mRNA levels in coronal mouse brain sections (**Study III**). Hybridization was carried out using complementary oligoprobes labeled with [α-33P] dATP. Briefly, air dried sections were incubated with hybridization cocktail in a humidified chamber for 16-18 h at 42°C. After hybridization, the sections were washed and dehydrated using alcohol. The sections were then developed using Kodak D19, fixed in Kodak Unifix and mounted. In order to check probe specificity, adjacent sections were incubated with an excess (100x) of unlabeled probe. This method enables us to localize and measure mRNA expression of a specific target in specific regions of the mouse brain. It preserves the integrity of the cells in the tissue section. Varying sensitivity of the available probes and long time required for developing signal are the caveats.

3.5.7 Immunohistochemistry

This technique was used to analyze the protein expression of COX-1/2, mPGES-1 in mouse brain sections (**Study III**) and α7nACh receptor on activated human astrocytes (**Study IV**). Briefly, the protein of interest is detected on paraformaldehyde fixed mouse brain sections by using polyclonal or monoclonal primary antibodies raised in rabbit, goat or rat. Later, the unbound antibodies are washed and the bound primary antibodies are recognized by using biotinylated secondary antibodies raised against them. Following that, an avidin-biotin-peroxidase complex is used to amplify the signal. The detection mixture contains hydrogen peroxide and chromagen 3,3'-diaminobenzidine tetrahydrochloride (DAB) which later develops into brown color (positive signal). The unspecific binding was averted by blocking the endogenous peroxidase, avidin and biotin activity. The specificity of the primary and secondary antibodies was confirmed using appropriate isotype control and by excluding primary antibodies accordingly. The main advantages of this method are able to detect the protein of interest at the cellular level, signal amplification, long lasting signal compared to fluorescent dyes and greatly helps to analyze region specific differences. The disadvantages are possibility of getting a high background, requires optimization and difficult to study co-localization. We minimized background by incorporating blocking steps for avidin, biotin and endogenous hydrogen peroxidase and used immunofluorescence to study co-localization.

3.5.8 Indirect immunofluorescence

This method was used to detect the protein expression of choline acetyltransferase (ChAT) enzyme in mouse splenocytes (**Study II**), to co-localize mPGES-1 and GFAP expression in mouse brain sections (**Study III**) and GFAP and α7nAChR expression in activated human astrocytes (**Study IV**). The detection was either performed using primary antibodies directly conjugated to fluorophores or using biotinylated secondary antibody -streptavidin detection system. This technique offers ease of multiplexing, fewer steps and wide dynamic range compared to immunohistochemistry. Lower sensitivity and photobleaching are the main disadvantages.

3.5.9 Enzyme Immunoassay

This method was used to quantify the PGE_2 content in culture supernatants (**Study IV**). The assay employs a forward sequential competitive binding technique where the analyte competes with HRP-conjugated PGE_2 for specific number of binding sites on monoclonal antibody bound to the plate. The HRP activity is then measured using an appropriate substrate at 450nm. The pros are simple to perform, sensitivity is 41.4 pg/ml, broad detection range is 39 - 2500 pg/ml and less cross reactivity. Lack of multiplexing for other PGs in the same sample, lesser sensitivity, accuracy and specificity compared to LC-MS/MS are the cons of enzyme immunoassay.

3.6 VAGUS NERVE STIMULATION

In our experiments, we carefully isolated the left cervical vagus nerve and electrically stimulated it for 5 mins. The stimulation parameters (1Hz for 2ms at 5V) used are in accordance with the previous studies demonstrating cytokine inhibition (29). We neither studied the electrical current dose response nor stimulation time dependent effects on inflammation. In contrast to earlier studies on VNS effects in acute inflammatory models, we chose to collect samples after 6 hours, a time point when both cytokine and prostaglandins are known to be elevated (5, 29). We induced endotoxemia in mice at a lower dose of LPS (2mg/kg) for the following reasons. It was ethically unacceptable to use the original lethal dose of 15mg/kg and keep the animals for 6 hours and on the other hand, a high dose of LPS could potentially mask effects of prostaglandin and thus make it unsuitable for our studies. We have shown that this modified LPS dose is adequate to cause sufficient proinflammatory cytokine release and most importantly, immunosuppressive effects of VNS were still evident after 6 hours of treatment in our endotoxemic model (114).

3.7 mPGES-1 GENE DELETION AND PHARMACOLOGICAL INHIBITION

To understand the role of mPGES-1 in VNS mediated CAP, we used in bred DBA/1lacJ mice with Ptges gene deletion (mPGES-1 -/-) and congenic wild type as controls (mPGES-1 +/+) (**Study I and II**). Mouse Ptges gene is composed of 40.4 kilobases inclusive of three exons

located on chromosome 2 and shares 84% homology with its human counterpart. Homozygous littermates were obtained by crossing heterozygous (mPGES-1 +/-) males and females and genotypes were later confirmed by PCR. Mice with mPGES-1 gene deletion are viable, reproduce and develop normally in comparison to mPGES-1(+/+) (90). No differences were spotted in general behavior, appearance, weight and hematological factors. In addition, deletion of mPGES-1 gene had no effect on the expression of COX-2 and other prostaglandin synthesizing enzymes (90, 115), blood pressure and thrombogenesis (116). Though no differences were detected among the constitutive prostanoid levels, LPS treatment resulted in significant suppression of PGE_2 levels and shunting was seen towards thromboxane B_2 (TxB_2) and PGD_2 metabolite in mPGES-1(-/-) compared to mPGES-1(+/+) macrophages. Interestingly, mPGES-1 gene depletion did not affect the total fatty acid composition in activated cells and brain tissue but induced some changes in inflamed treated spleen(117).

Pharmacological inhibition of mPGES-1 activity was carried out by using Compound III, a benzoimidazole derivative (**Study II**). Compound III inhibits both human and rat recombinant mPGES-1 and displays high selectivity towards mPGES-1 among other prostanoid synthases. Under stimulated conditions, mPGES-1 inhibition significantly decreased PGE_2 and increased prostacyclin metabolite 6-keto-$PGF_{1\alpha}$ *in vitro*. Intriguingly, in the inflammatory air pouch model, while both mPGES-1 gene deletion and inhibition failed to affect exudate volume, they elicited opposing effects on the cellular infiltration. Moreover, while the excess PGH_2 resulting from mPGES-1 deletion caused shunting to TXB pathway, inhibition of its enzymatic activity did not affect other prostanoids *in vivo* (118).

3.8 STATISTICAL ANALYSES

All the statistical tests were done using Graph Pad Prism 6.0 software.

Study I

Data are represented as mean±standard error mean (SEM) for all samples and analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test for multiple comparisons. Statistical significance was set at P values less than 0.05.

Study II

All samples were run in duplicates and data are represented as mean±SEM. Statistical analyses were performed using one-way ANOVA (Bonferroni's post hoc test) or Student's T–test. P values ≤ 0.05 were considered as statistically significant.

Study III

All values are represented as mean ±SEM and analyzed using Student's T-test or one-way ANOVA (Bonferroni's post hoc test). Differences between treatment groups were considered to be significant for P values less than 0.05.

Study IV

Samples were run in duplicates and reported as mean±SEM. Data was analyzed using one-way ANOVA (Bonferroni's post hoc test) and P values ≤ 0.05 were considered as statistically significant.

3.9 ETHICAL CONSIDERATIONS

Animals were fed and maintained under temperature controlled, pathogen free conditions as per the guidelines of care and use of animals. Mice were allowed to acclimatize to our facility for a minimum of 1 week prior to the onset of experiments at constant room temperature and a 12 hr light/dark cycle. All procedures were performed and approved by the Regional Ethics Committee at Karolinska Institutet, Sweden.

4 RESULTS AND DISCUSSION

Study I.

mPGES-1 dependent PGE_2 is crucial for the complete functioning of the cholinergic anti-inflammatory pathway in endotoxemia

PGE_2 is known to play a central role in the bidirectional communication between central nervous and immune system, especially during peripheral inflammation. Onset of sickness syndrome and HPA axis, induction of pain and activation of EP3 (PGE_2 receptor) bearing cells in the vagal nuclei in response to systemic inflammation (6) has clearly demonstrated the indispensable role played by PGE_2 in neuro-immune interactions and possible involvement in immunoregulatory mechanisms. In fact, administration of PGE_2 in the CNS resulted in the neuronal stimulation of specific brain structures including NTS, a converging point for afferent vagus nerves (119). This prompted us to question the role of PGE_2 in association with immunosuppressive effects of VNS. Since inflammation induced PGE_2 synthesis is mainly attributed to the terminal enzyme mPGES-1 (120), we chose to study the outcome of VNS on LPS induced cytokine production in mPGES-1 (+/+) and mPGES-1 (-/-) mice *in vivo*.

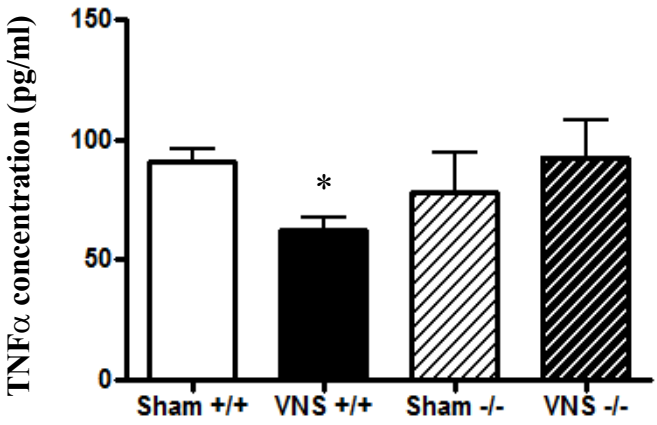
In 2000, Borovikova and colleagues (121) clearly demonstrated the attenuating effects of electrical stimulation of vagus nerve on the TNFα synthesis in endotoxemic adult rats. In accordance with their results, we observed that VNS resulted in the strong immunosuppression in endotoxemic mPGES-1(+/+) mice compared to SHAM (LPS+ neck surgery only). VNS treatment significantly inhibited TNFα, IL-1β, IFNγ and IL-12 in the serum. Importantly, VNS limited TNFα production in the spleen, a major target organ in LPS induced inflammation and CAP. Intriguingly, in the case of mPGES-1 (-/-), VNS failed to hamper the production of proinflammatory cytokines both in the serum (**Figure 5**) and spleen. Importantly, in line with Uematsu et al., (89) deletion of mPGES-1 gene did not impede the upregulation of any of the LPS induced cytokines both in the serum and spleen. This helped us to conclude that the failure of VNS to suppress inflammation in mPGES-1(-/-) mice is solely due to absence of mPGES-1 dependent PGE_2 production and cannot be attributed to a general cytokine release impairment related to the genotype.

Next, the PGE_2 production in response to VNS/SHAM treatment was investigated in the spleen and brain respectively. Interestingly, VNS did not increase PGE_2 levels in the endotoxemic mouse spleen in mPGES-1(+/+). On the other hand, as expected, the mPGES-1 gene deletion in mPGES-1(-/-) mice abolished nearly 80 % of the PGE_2 release in response to LPS with no major differences between SHAM and VNS treatment. In parallel, apart from similar observations in homogenized mouse brains with regard to mPGES-1 dependent PGE_2 synthesis, we could detect a significant suppression of PGD_2 in both SHAM and VNS of mPGES-1 (-/-) mice.

Local PGE_2 has been shown to increase ChAT and ACh production through EP4 activation in phytohaemagglutinin (PHA) treated human leukemic cell line (MOLT-3) (110). In corroboration with these findings, VNS induced ACh release was evident in mPGES-1 (+/+) spleen, however such an effect was abrogated in mPGES-1(-/-) mice as shown in **Figure 6**. Thus, we could conclude that, in response to VNS, mPGES-1 (-/-) mice displayed both a defective immune suppression and lack of the earlier observed peak in VNS-induced acetylcholine production. These findings thus, not only implicated the importance of PGE_2 for the optimal functioning of CAP but also raised several research questions that were further addressed in Study II and III.

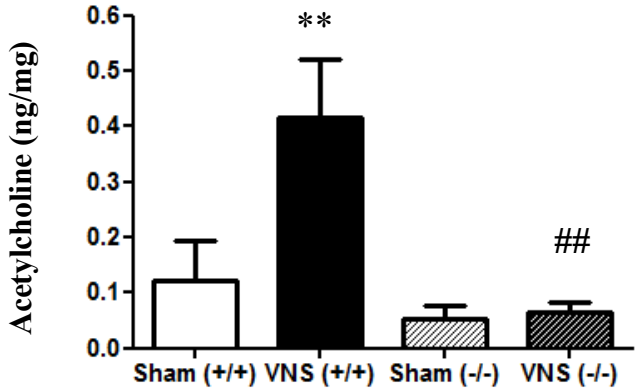
It is important to note that activation of CAP by VNS is not only restricted to electrical stimulation of efferent vagus nerve but also sends action potentials along afferent nerve fibers (122). Moreover, while the series of neuro-immune events orchestrated by efferent vagus activation is well categorized (123), the potential effects of afferent VN on the CNS remains elusive. PG profiling on brain and spleen homogenates was instrumental in confirming the inhibition of PGE_2 synthesis in mPGES-1(-/-) mice, however by using this method potential region-specific differences related to VNS in mPGES-1(+/+) brain could be lost. In order to understand the impact of mPGES-1 depletion on CAP in detail, we decided to study the already elucidated CAP related events in mPGES-1(-/-) mouse spleen (Study II) and examine the effects of VNS on PGE_2 related enzymes in specific regions of the endotoxemic mouse brain (Study III).

Figure 5. Cytokine production in mPGES-1 (+/+) and (-/-) mouse serum after SHAM and VNS treatment.



All animals have received LPS (2mg/kg) injection. VNS induced suppression of TNFα was abolished following mPGES-1 gene deletion. All values are represented as mean ± SEM and *p<0.05 (One-way ANOVA) denotes statistical differences between VNS and corresponding SHAM mice (n=6-7 per group).

Figure 6. ACh release in mPGES-1 (+/+) and (-/-) spleen extracts after SHAM and VNS treatment.



VNS increased ACh content compared to SHAM in mPGES-1 (+/+) spleen. In contrast, mPGES-1 deficiency impaired the ACh release in response to VNS. Data are expressed as ng/mg of spleen. **p<0.01 (One-way ANOVA) compared to SHAM (+/+); ## compared to VNS (+/+) (n=6 per group).

Study II

mPGES-1 gene deletion impairs the neuro-immune circuitry of CAP in the endotoxemic mouse spleen

Detailed mechanistic studies on CAP have shown activation of the splenic nerve by VNS to release sympathetic neurotransmitter NE in juxtaposition to β_2 adrenergic receptor (AR) expressing $CD44^{hi}$ $CD62L^{lo}$ effector memory T cells residing in the white pulp of spleen (25). Based on our current understanding of CAP, defective ACh synthesis in response to VNS treatment following mPGES-1 genetic deletion, in **Study 1**, could be an outcome of (i) insufficient splenic nerve activity, (ii) defective expression of β_2 adrenergic receptors or (iii) setbacks in the cholinergic synthesis machinery.

In mice, intravenous LPS treatment has been shown to increase the sympathetic splenic nerve activity and such an effect is believed to be mainly mediated by the central PGE_2 synthesis (10). In our experiments, though the splenic nerve activation is directly linked to VNS, given the fact that mPGES-1 gene deletion significantly reduced brain PGE_2 levels, we wanted to verify if it could affect the splenic NE release in response to VNS in mPGES-1(-/-) mice. On the contrary, VNS induced splenic NE release was intact despite the depletion of mPGES-1 dependent PGE_2 expression as depicted in **Figure 7**. This result helped us to rule out the possible involvement of brain PGE_2 in affecting splenic nerve activation associated with VNS and pointed out to the possibility of mPGES-1 gene deletion affecting other molecular events downstream of NE release in CAP.

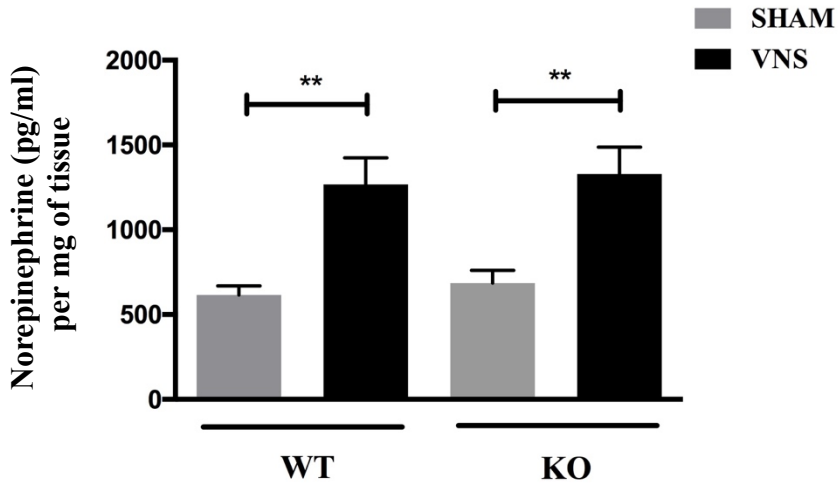
Recent studies on β_2 AR (-/-) mice, proved the importance of the β_2 adrenergic receptor in executing the immunoprotective effects of VNS (12). We examined the β_2 adrenergic receptor expression on $CD\ 44^{hi}$ $CD\ 62L^{lo}$ effector memory cells among the spleen lymphocyte population following VNS treatment. In comparison to mPGES-1 (+/+), mPGES-1 gene deletion did not affect the naïve ($CD44^{lo}$ $CD62L^{hi}$), effector ($CD44^{lo}$ $CD62L^{lo}$), central memory ($CD44^{hi}$ $CD62L^{hi}$) or effector memory ($CD44^{hi}$ $CD62L^{lo}$) populations following VNS in LPS treated mouse spleen. Moreover, no significant differences were observed in β_2 adrenergic receptor expression between the VNS treated mPGES-1(+/+) and (-/-) effector memory cell populations. Thus, mPGES-1 depletion altered neither the proportion of effector memory population nor the expression β_2 adrenergic receptors on their cell surface.

LPS activation of splenocytes prepared from both mPGES-1 (+/+) and (-/-) mouse resulted in high concentrations of proinflammatory mediators such as TNFα, IL-6 and KC GRO in culture supernatants. Interestingly, treatment of activated mPGES-1 (+/+) splenocytes with NE mitigated the cytokine synthesis. However, such limiting effects of NE were strongly altered in case of TNFα and negated in IL-6 and KC GRO in the absence of mPGES-1 expression. Unlike neurons, immune cells cannot store and release ACh instantly upon activation and instead produce it by the enzymatic action of choline acetyl transferase (ChAT) on choline (59). As shown earlier by Vijayaraghavan et al. (124), LPS activation

increased ChAT protein levels in mPGES-1 (+/+) splenocytes. On the contrary, though mPGES-1 (-/-) splenocytes expressed relatively similar ChAT expression under resting conditions, it failed to augment its protein levels upon LPS stimulation as shown in **Figure 8**. Intriguingly, mPGES-1 gene deletion completely abolished the mitigating effects of nicotine treatment in LPS activated splenocytes. Translational experiments involving pharmacological inhibition of mPGES-1 function in LPS stimulated human PBMC led to upregulation of TNFα synthesis. In line with mouse splenocyte data, nicotine's immunomodulatory effects on activated human PBMC were negated by mPGES-1 inhibition. These findings complement the numerous studies that suggest involvement of PGE_2 in the cholinergic limiting effects on destructive cytokine release (107, 108).

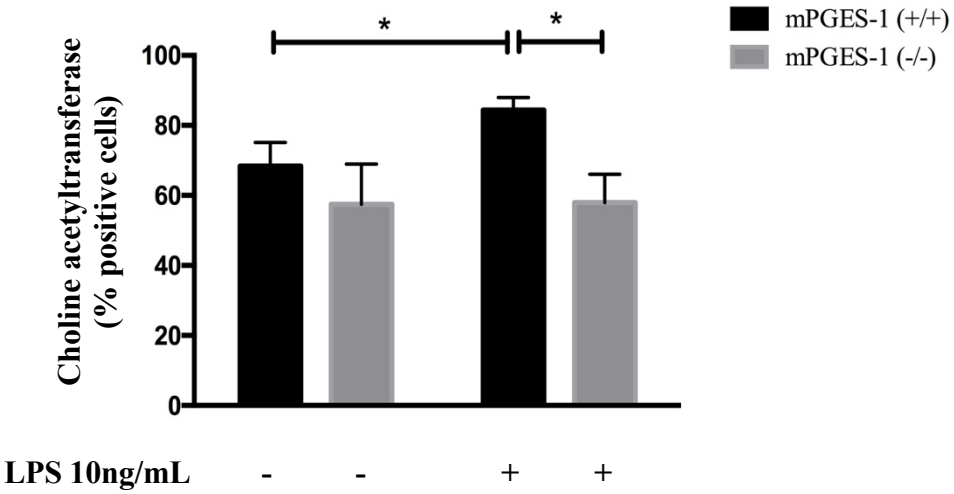
We demonstrate here for the first time that central mPGES-1 dependent PGE_2 synthesis is not crucial for VNS mediated splenic nerve activation and subsequent NE release. On the contrary, we observed a strong involvement of mPGES-1 dependent PGE_2 in cholinergic synthesis and immune regulation of activated immune cells in the spleen. In conclusion, our data implicate the importance of mPGES-1 dependent PGE_2 in cholinergic synthesis and regulation of immune responses following CAP activation in mouse spleen as depicted in **Figure 9**.

Figure 7. Splenic norepinephrine release in mPGES-1 (+/+) (WT) and (-/-) (KO) animals following VNS.



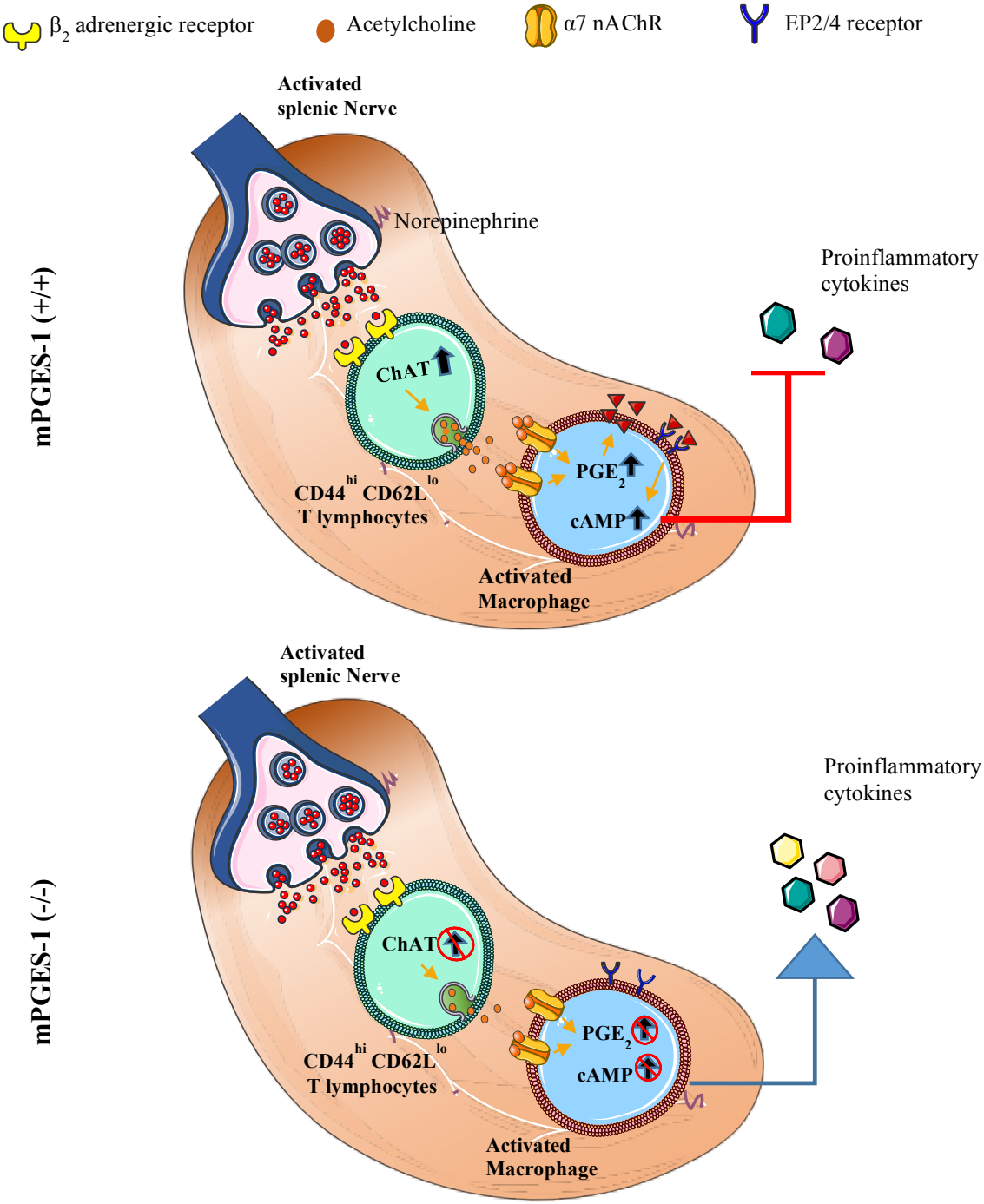
Electrical stimulation of the VN increased splenic NE content by ~ 2-fold irrespective of the presence or absence of mPGES-1 expression. All values are represented as mean±SEM from 4-5 individual animals per treatment (**p<0.01; SHAM versus VNS; One-way ANOVA)

Figure 8. Choline acetyltransferase expression in LPS stimulated mPGES-1(+/+) and (-/-) splenocytes



Unstimulated mPGES-1(+/+) and (-/-) splenocytes displayed similar levels of ChAT expression. LPS increased percentage of ChAT positive cells among mPGES-1(+/+) splenocytes (* p<0.05; LPS versus control within mPGES-1(+/+); Mann-Whitney Test). On the contrary, LPS failed to increase ChAT expression mPGES-1 depleted splenocytes (*p<0.05; mPGES-1(+/+) versus (-/-) within the LPS treatment group; Mann-Whitney Test). All values are represented as mean±SEM from two independent experiments.

Figure 9. Schematic diagram of VNS mediated event in mPGES-1 (+/+) and (-/-) spleen



In an endotoxemic spleen, VNS induces splenic nerve (SN) NE release adjacent to $CD44^{hi}$ $CD62L^{lo}$ T cells resulting in β2 AR stimulation and ACh synthesis. Subsequent α7 nAChR interaction on activated macrophages increases endogenous PGE_2 and cAMP through EP2/4 activation, thereby controlling inflammation. In our current study, we demonstrate that in mPGES-1 gene deleted mice, VNS induced sympathetic SN activation and β2AR expression on effector memory cells are intact. However, choline acetyltransferase (ChAT) dependent ACh release and inhibition of cytokines in response to nicotine (α7nAChR agonist) are impaired, thereby clearly demonstrating the role of mPGES-1 dependent PGE_2 as a crucial mediator in the cholinergic processes related to VNS.

Study III

Treatment of endotoxemia by vagus nerve stimulation increases mPGES-1 expression and limits substance P neuropeptide synthesis in mouse brain

Data from Study I showed that immunosuppressive effects of VNS were highly compromised in endotoxemic mice lacking mPGES-1 expression and VNS had no effect on the PGE_2 protein levels of LPS inflamed spleen under the described experimental conditions. At the same time, upregulation of PGE_2 synthesis across brain endothelial cells and parenchyma including vagal nuclei induced by systemic inflammation and its role as neuro-immune mediator in initiating sickness syndrome and HPA axis has been well studied (6, 125). In addition, stimulation of VN can increase brain cholinergic activity through afferent vagus activation (54) and limit COX-2 expression in specific brain regions (126). This raises an interesting research question of the potential effects of VNS on PG system in endotoxemic brain. Thus, we aimed to study the effects of VNS on the brain PGE_2 related enzymes (COX-1/2 and mPGES-1) and substance P expression in mouse brain during endotoxemia using *in situ* hybridization and immunohistochemistry.

We identified specific brain regions that were activated by acute VNS by measuring the expression of the neuronal activation marker, c-Fos. We observed strong c-Fos activity in hippocampus, hypothalamus, cingulate cortex, periaqueductal grey (PAG), and dorsal raphe nucleus (DRN) which are proven to be associated with memory, stress, mood, pain and serotonergic neurotransmission (127). Interestingly, acute VNS resulted in significant enhancement of LPS induced c-Fos expression in hippocampus and hypothalamus. On the contrary, in agreement with Cunningham et al., VNS did not affect LPS induced c-Fos levels in cingulate cortex and DRN. However, chronic VNS has been reported to activate ΔFos, a c-Fos metabolite, in both regions mentioned above (127). VNS also strongly inhibited the LPS induced brain substance P expression in all regions studied except the DRN, which explain the anti-nociceptive effects of VNS in migraine(128).

Immediate release of PGE_2 in response to injury or infection is generally fueled by COX-1 enzymatic action followed by COX-2 during the later stages of an immune response (129). In contrast, brain inflammation is mostly associated with COX-2 activity (130). In support of this, we observed a significant increase in COX-2 protein levels in hypothalamus, PAG and DRN following LPS treatment. In contradiction to the recent reports on VNS resulting in decrease of COX-2 mRNA in the cortex (126), we did not detect any significant differences in LPS treated brain COX-1 and 2 expression following VNS. It is noteworthy to mention that a time dependent analysis is required to better understand the VNS effects on COX enzymes.

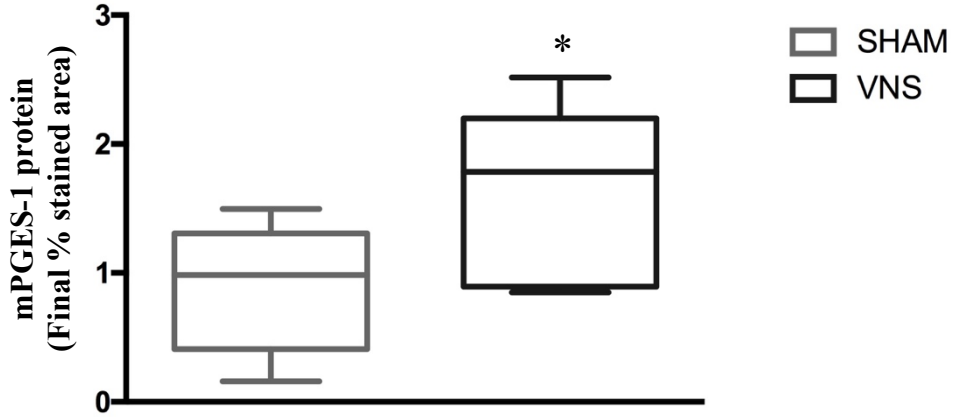
Contradictory findings exist concerning the constitutive expression of mPGES-1 in the brain. Engblom et al have reported basal mPGES-1 expression to be rare and restricted to specific regions in the rat brain parenchyma (125). However, in support of Eskilsson and colleagues

(96), we detected constitutive mPGES-1 expression in all regions examined. In addition, LPS treatment alone did not alter the constitutive expression of mPGES-1 in the mouse brain in any way. These differences, however, could be species dependent and needs further investigation. Intriguingly, in connection with our earlier results (Study I) suggesting mPGES-1 dependent PGE_2 involvement in CAP, VNS increased mPGES-1 protein level in the hypothalamus (**Figure 10**) and displayed a tendency to increase mPGES-1 expression in other regions such as cingulate cortex, PAG and DRN. Interestingly, in line with previous studies (96) reporting astrocytic mPGES-1 expression, we observed mPGES-1 co-localization with GFAP positive astrocytes in the hypothalamus, hippocampus, cortex and along blood vessels in VNS treated inflamed mouse brain.

In another set of experiments carried out to study the impact of PG inhibition on neuroinflammation, LPS treatment for 2 hours increased the release of IL-6, IL-1β and CXCL1 in both brain and spleen homogenates. Intriguingly, intrathecal COX inhibition further increased the above-mentioned cytokines to significant levels in the brain. On the contrary, intraperitoneal treatment of the same COX inhibitor at a dose 10 times higher than i.t did not change the LPS induced brain cytokine production. Interestingly, both systemic and central COX inhibition had no effect on the LPS induced cytokines in the spleen.

Thus, our data illustrates that electric stimulation of VN is associated with activation of specific regions of mouse brain. Increase in hypothalamic mPGES-1 expression in response to VNS further reiterates the interdependence between cholinergic and PGE_2 systems in immune regulation. Finally, our COX inhibition results hint at the possible involvement of COX dependent PGs in brain cytokine production following peripheral inflammation.

Figure 10. Hypothalamic mPGES-1 protein expression in endotoxemic mouse brain following VNS



No significant differences were observed in the mPGES-1 expression between SHAM and VNS treatments among hippocampus, CC, PAG and DRN, however VNS strongly increased mPGES-1 protein level in the hypothalamus of LPS treated mice. (*P<0.05; n= 7; Student's T-test)

Study IV

Immunomodulatory effects of the cholinergic agonist nicotine on activated human astrocytes - role of PGE_2

Endotoxemia has been associated with increased IL-1β and IL-6 release by activated astrocytes in the hypothalamus (131). Interestingly, activation of the CAP by electrical stimulation of the vagus nerve increases brain ACh levels mediated by the adrenergic activation of the central cholinergic network (54) and has been shown to reduce neuroinflammation (126). Binding of ACh to its nicotinic receptor (α7 in particular) on innate immune cells of CNS such as microglia has anti-inflammatory and neuroprotective effects (34). On the other hand, such cholinergic effects on astrocytes, most abundant glial cells, remains unclear. Suppressive effects of VNS on neuroinflammation (54), co-localization of mPGES-1 and GFAP in VNS treated brain regions (Study III), interdependence between cholinergic and PGE_2 systems (Study I) motivated us to study the effects of nicotine on IL-1β activated human astrocytes and role of PGE_2 in the cholinergic immune modulation of neuroinflammatory events *in vitro*.

Over 90% of the cultured human astrocytes grown *in vitro* displayed strong GFAP expression. Activation of 80-90% confluent, serum starved human astrocytes with recombinant IL-1β (10ng/ml) induced IL-6, TNFα and IL-8 production. IL-1β neither induced Th2 cytokine release nor affected IL-10 levels in culture supernatants. Interestingly, in line with previous observations, simultaneous treatment of human astrocytes with IL-1β and nAChR agonist, nicotine (1, 10 or 100μM) for 20 hours significantly downregulated IL-1β induced IL-6 levels. In addition, high dose of nicotine (100μM) also limited the production of other crucial mediators of inflammation such as TNFα, IL-1β and IL-8 respectively. We propose that nicotine executes its effects through α7nAChR whose expression was evidently detected on activated astrocytes.

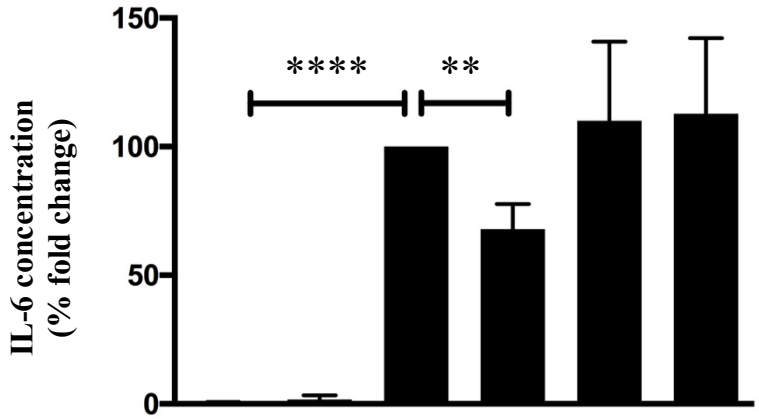
Acetylcholine esterase (AChE) expression is usually detected in neurons and barely expressed in astrocytes except astrocytic tumors (132, 133). In line with this, we did not detect any measureable levels of AChE expression in activated human astrocytes. In contrast, butyrylcholinesterase (BuChE) is closely associated with inflamed astrocytes and inhibitory effects of ACh on BuChE are reported (134). In line with previous reports(135), we found that high dose of nicotine reduced BuChE protein synthesis by activated astrocytes.

Upregulation of COX-2 enzyme and subsequent release of PGE_2 in response to nicotine is documented in microglia, monocytes and other immune cells (108, 136). *In vitro* studies by Takahashi et al (136) in activated monocytes attributed the beneficial effects of nicotine to COX-2 dependent PGE_2 release and subsequent EP2/4 receptor activation resulting in high concentrations of the secondary messenger, cAMP. In concordance with previous studies on microglia (108), COX-2 inhibition by NS-398 treatment reversed immunoregulatory effects

of nicotine on activated human astrocytes with respect to IL-6 production as clearly shown in **Figure 11**.

These results thus confirm the possible beneficial effects of cholinergic activation of nicotinic receptors on neuroinflammation driven by activated astrocytes. Furthermore, they reveal the cross-talk that exists between cholinergic and prostaglandin systems during immune regulation as illustrated in **Figure 12 and 13**.

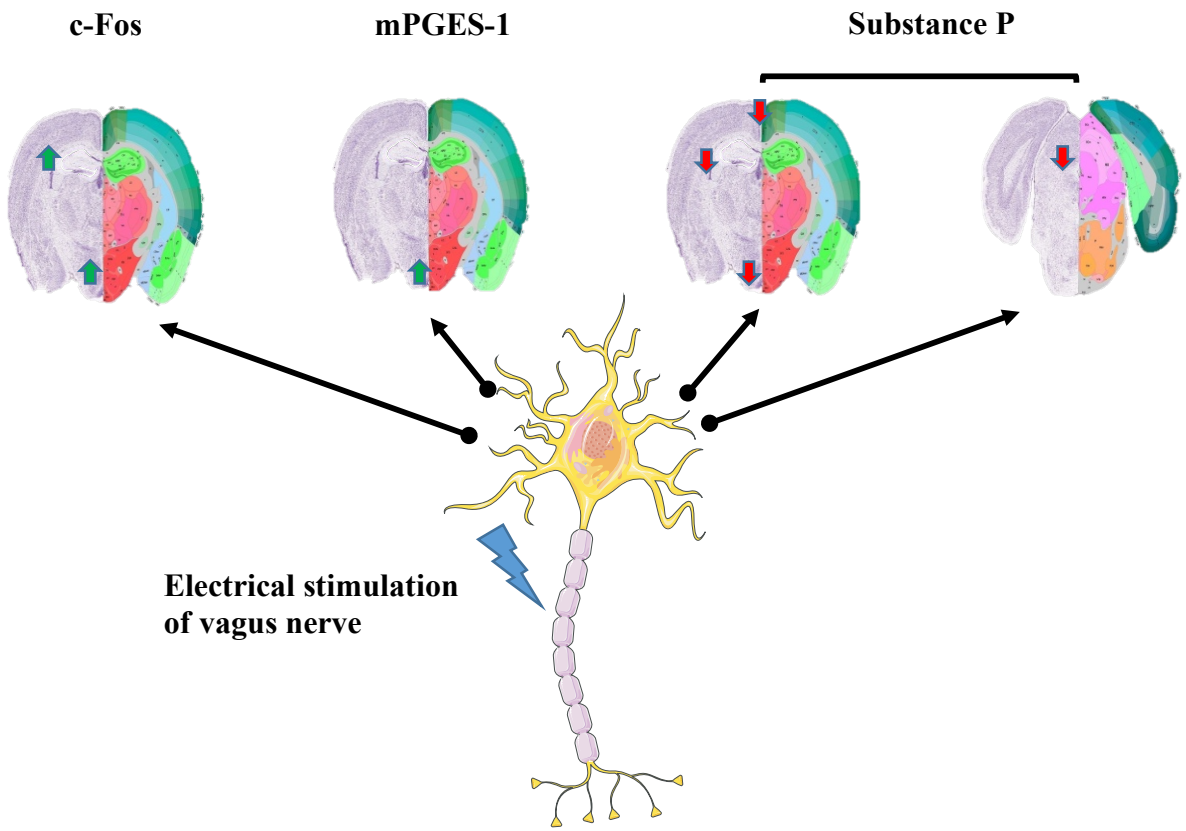
Figure 11. Effect of COX-2 inhibition on the nicotine induced IL-6 reduction in activated human astrocytes



IL-1β (10ng/ml)	-	-	+	+	+	+
Nicotine (10μM)	-	-	-	+	+	+
NS398 (μM)	-	-	-	-	**1**	**10**
DMSO (0.5%)	-	+	+	-	-	-

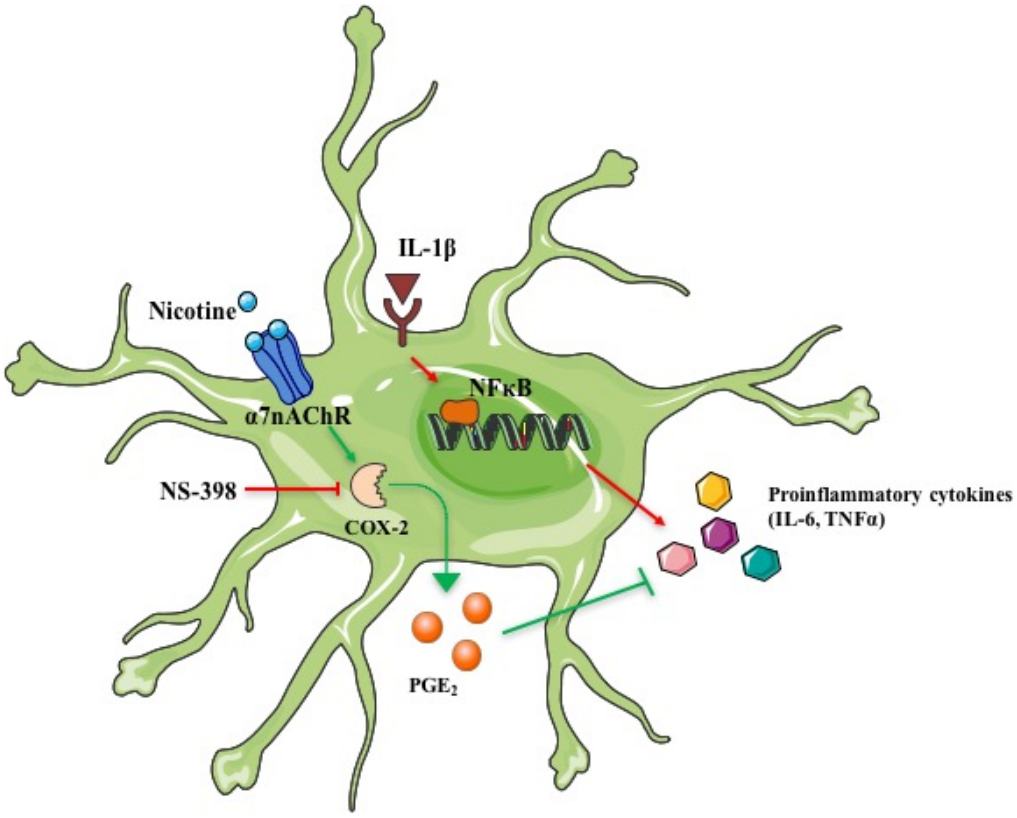
Cyclooxygenase-2 activity in human astrocytes was inhibited by using NS-398 (1 and 10μM). Nicotine treatment significantly downregulated IL-6 release by activated astrocytes. Intriguingly, NS-398 completely blocked IL-1β induced PGE_2 synthesis and reversed the immunomodulatory effects of nicotine on IL-6 release. COX-2 inhibitor did not elicit any effects on IL-6 production on its own. All values are represented as mean±SEM in terms of fold change compared to cytokine level following IL-1β treatment. Statistical analysis was performed using non-parametric Student t-test (**** ($p<0.0001$) & ** ($p<0.01$))

Figure 12. Schematic representation of the central effects of VNS on endotoxemic mouse brain



Acute VNS strongly activated specific regions of the brain such as hippocampus and hypothalamus and increased hypothalamic mPGES-1 protein expression in mice with systemic inflammation. On the contrary, VNS did not alter COX-1/2 expression. mPGES-1 expression co-localized with GFAP+ astrocytes in specific regions of brain including the hypothalamus. Interestingly, stimulation of vagus nerve resulted in suppression of substance P expression in specific regions of the brain including PAG.

Figure 13. Schematic representation of the role of PGE_2 in mediating the immunomodulatory effects of nicotine during reactive astrogliosis.



Neuroinflammatory responses involving cytokines such as IL-1β upregulate NF-kB activity and promote NF-kB dependent cytokine production such as IL-6, TNF-α etc. In our study, nicotine inhibited production of IL-1β stimulated inflammatory cytokines through COX-2 pathway to restore homeostasis. Binding of nicotine to its α7nACh receptor on reactive astrocytes increases COX-2 dependent PGE_2 release that seems to be a crucial mechanism in mediating cholinergic immunomodulation.

5 GENERAL DISCUSSION

The classical lipid mediator, PGE_2, has been linked to multiple pathological processes involved in acute and chronic inflammatory diseases (137). Of the three terminal synthases known, mPGES-1 is mainly responsible for PGE_2 upregulation and subsequent trafficking of innate immune cells at the inflamed site during the initial phase of inflammation (87). Numerous *in vivo* and *in vitro* studies have demonstrated the beneficial outcomes associated with reduced mPGES-1 activity in controlling inflammation (90, 138). Thus, pharmacological inhibition of mPGES-1 is looked upon as a promising strategy for treating inflammatory conditions and efforts are underway to develop specific mPGES-1 inhibitors (88). At the same time, considerable number of studies have emerged in the recent past focusing on the importance of PGE_2 in regulating immune responses and initiating resolution of inflammation (94, 95, 102, 139).

Strong evidence has emerged advocating the precedence of high levels of PGE_2 to induction of special pro resolving mediators (SPMs). SPMs such as lipoxins, resolvins and protectins have been identified to be instrumental in orchestrating the series of events leading to resolution of inflammation (97). Detailed studies in mice have implicated PGE_2 to activate the transcription of SPMs, thereby driving the lipid mediator switch from the production of proinflammatory leukotrienes (LTB_4) to pro resolving mediators (140). Intriguingly, Mikaraj et al, clearly demonstrated the link between vagus nerve and SPMs where vagotomy increased the proinflammatory cytokines, reduced the levels of pro resolving mediators and delayed the resolution of inflammation (141). In this context, the failure of VNS to limit the production of LPS induced proinflammatory cytokines in serum and spleen following mPGES-1 gene deletion (**Study 1**) provides convincing evidence in support of the importance of PGE_2 in vagus nerve mediated resolution.

ACh producing T cells are critical for functioning of the distal part of CAP (25) and activation of EP_4, one of the four PGE_2 receptors, has been illustrated to increase ChAT mediated ACh release in activated human leukemic T cells (110). Abolishment of VNS induced ACh release in mPGES-1 depleted mice (**Study 1**) further confirms the crucial role of PGE_2 on release of ACh upon cholinergic nerve stimulation. Interestingly, therapeutic effects of cholinergic and adrenergic drugs in reducing intraocular pressure in glaucoma patients are attributed to PGE_2 (142). PGE_2 involvement in cholinergic mechanisms is also extended to ACh induced smooth muscle contraction where use of NSAIDS inhibited nicotine/ACh induced guinea-pig ileum contractions and reversed by PGE_2 replenishment (143). Intriguingly, anti-inflammatory effects of nicotine have also been shown to be mediated through COX-2/PGE_2 induction (136, 144). Our observations reiterate the participation of PGE_2 in executing nicotine immunomodulatory effects in activated immune cells both in mice and humans and further uncover the previously unknown involvement of mPGES-1 in mediating PGE_2 effects on cholinergic immune regulation (**Study II**).

Parallel to the suppressive effects of efferent CAP in the periphery, studies have proposed the possibility of an afferent CAP mechanism in the CNS where α7nAchR activation on inflamed microglia reduced its cytokine release (35, 54, 145-147). Interestingly, induction of acute hypoxic insult in near-term ovine fetus increased vagal activity and α7nAchR expression leading to decreased HMGB1 translocation in activated microglia in specific regions of the brain (148). Further evidence stems from Meneses et al, where they demonstrated the therapeutic ability of VNS in limiting brain cytokine release and microglial Iba-1 expression in endotoxemic mice (149). Fresh evidence supporting the existence of an afferent CAP is provided by the activation of specific regions such as hippocampus and hypothalamus in mouse brain following acute VNS (**Study III**).

The fact that VNS upregulated mPGES-1 in hypothalamus (**Study III**) is in line with reports by Schweighögfer et al demonstrating VNS mediated downregulation of hypothalamic IL-6 and CXCL1 mRNA expression in LPS treated mouse brain (126). Together with the reported upregulation of hypothalamic IL-6 levels in LPS injected mPGES-1 (-/-) mouse (150, 151). These data signify a possible protective role of mPGES-1 dependent PGE_2 on the LPS induced proinflammatory cytokine expression in mouse brain. Furthermore, co-localization of mPGES-1 and GFAP in hypothalamus implicates astrocytes to be a potential source of PGE_2 synthesis in response to VNS. We also touched upon the overall effects of COX mediated PGs on neuroinflammation where, in support of Blais et al (152), COX inhibition enhanced the neuroinflammatory cytokine release triggered by systemic endotoxin. However, we have not yet studied the effects of specific mPGES-1 inhibition in this context and the exact mechanisms of mPGES-1 in mediating the cholinergic effects of CAP on neuroinflammation need further investigation.

Cholinergic deficit is one of the main pathological consequence of Alzheimer's disease and enhancing cholinergic levels in AD patients has been shown to slow the disease progression (151, 153). Interestingly, observational studies in an elderly cohort linked heavy long term use of NSAIDS to increased incidence of dementia and AD (154) and further investigations are carried out to understand the underlying mechanism. Neuroprotective effects of nicotine in reducing Aβ accumulation, inhibiting NF-κB in mice (155) and limiting TNFα release by activated microglia (145) make it a potential therapeutic agent in alleviating AD progression. Nicotine exerted similar inhibitory effects on NFκB dependent cytokines released by reactive human astrocytes and most importantly, its limiting effects on IL-8, a neutrophil chemoattractant, marks the beginning towards resolution. In support of previous studies on PGE_2 dependent cholinergic mechanisms in the periphery and microglia (108, 136), use of COX-2 inhibitors (NS-398) had detrimental effects on nicotine's immunosuppression of reactive astrocytes (**Study IV**). PGE_2, thus, acts as crucial mediator in the use of cholinergic agents to reduce astrogliosis and control neuroinflammation. Whether NSAIDS or mPGES-1 inhibition can interfere the beneficial effects of VNS on neuroinflammation needs to be investigated.

There is growing body of evidence against COX/PGE_2 inhibition during inflammation claiming that these therapies might result in delaying the resolution process of self-limited inflammation (97-99). Use of NSAIDS stretched the time to resolve inflammation following tooth extraction (156) and impaired healing of gastric ulcers (157). This observation is not only restricted to acute inflammation but also seen in various chronic inflammatory conditions where COX inhibition affected resolution in murine collagen induced arthritis (98). COX-2 has also been illustrated to take part in the resolving acute inflammation in the brain (152), lung (158), liver (159) and colon (160) respectively. In a clinical study, use of NSAIDS did not improve the pannus formation in joints among patients suffering acute flare and remission (99). Interestingly, compared to short term users, long term use of diclofenac in patients was associated with increase of osteoarthritic damage in their hips and knee (78). Furthermore, inflammatory bowel disease relapsed in 40 % and 70 % of the patients within six weeks of coxib and celecoxib treatment (161). Similarly, 17-28 % of patients with Crohn's disease experienced a relapse after 4 weeks of NSAIDS (162). Oral dose of ibuprofen in human subjects taking a single session of resistance exercise not only inhibited the induction of PGs and leukotrienes post exercise, but also limited the generation of pro resolving mediators (163), thus explaining the ineffective muscle recovery in response to NSAIDS treatment. Several clinical studies have also reported the detrimental effects of NSAIDS on bone healing (80, 81). These observations thus urge us to revisit the role of NSAIDS in inflammation and their importance in programming resolution.

On the other hand, cholinergic mechanisms are proved to regulate cytokine production through multiple anti-inflammatory pathways and confer protection against uncontrolled inflammation (31, 38, 164). This has made VNS and other cholinergic agonists a promising therapy for a range of inflammatory diseases such as sepsis (39), ischemia (165, 166), pancreatitis, Alzheimer's(151), stroke(167) etc . Recent clinical trials on VNS in patients suffering from RA(64) and Crohn's disease (168) led to significant cytokine reduction and alleviated the disease severity towards remission. Intriguingly, the response rates were ~70 % in both types of patients. Having said that, recent findings of reduced pro resolving mediator synthesis and delayed resolution in vagotomized mice demand a deeper understanding of the cholinergic anti-inflammatory pathway and its effects on other biological systems can help us to develop a better therapeutic strategy. In line with this, our experiments shed light on the importance of PGE_2 in the optimal functioning of CAP and provide new knowledge on possible PGE_2-cholinergic cross talk in mediating immune regulation. We firmly believe that our study can serve as a model for expanding our knowledge on the role of prostaglandins in the underlying mechanisms and possible implications on VNS or specific alpha 7 nicotinic agonist therapies in treating diseases. Moreover, the role of PGE_2 in cholinergic signaling is largely unknown, and further knowledge may lead to alternative ways of activating the CAP, as well as more information on potential unwanted effects of drugs inhibiting prostaglandin formation, i.e. NSAIDs.

6 CONCLUSION AND FUTURE PERSPECTIVES

In this thesis, we have investigated the role of mPGES-1 dependent PGE_2 in cholinergic immune regulation mediated by CAP activation and cholinergic agents such as nicotine. mPGES-1 activity was either abolished by genetic deletion (**Study I, II**) or suppressed by pharmacological inhibition (**Study II**). Here, we showed the importance of mPGES-1 gene expression and subsequent PGE_2 production for VNS induced ACh release and subsequent optimal CAP functioning in controlling systemic inflammation (**Study I**). We interestingly found that mPGES-1 deficient mice displayed intact NE release in the spleen after activation of the CAP, thus confirming that PGE_2 does not play an important role for the neurosignaling part of the CAP. However, the distal part of the pathway, with the NE acting on T-cells stimulating ACh release was not functioning in mPGES-1 deficient mice, indicating that PGE_2 plays a crucial role in cholinergic synthesis by regulating the ChAT expression in immune cells (**Study II**). Intriguingly, mPGES-1 depletion affected both cholinergic immune regulation in both mouse and human immune cells (**Study II**). Interestingly, activation of CAP by VNS induced mPGES-1 expression in hypothalamus (**Study III**) and indicates a potential immunomodulatory role of PGE_2 in afferent CAP mechanism. In support of this view, suppression of proinflammatory cytokine release by activated astrocytes following nicotine treatment was reversed by COX-2/PGE_2 inhibition (**Study IV**).

Our studies thus help us to improve our understanding concerning the interaction between the cholinergic and prostaglandin systems in regulating key innate immune responses and also add novelty to the role of mPGES-1 induced PGE_2 in resolving inflammation. We firmly believe that our findings on the previously unknown functions of PGE_2 as a mediator in modulating our immune system will contribute towards choice of use of NSAIDS in patients receiving VNS treatment for inflammatory conditions and also for those undergoing remission. While pharmacological mPGES-1 inhibition has been advocated as a favorable therapeutic target to control inflammation, keeping the impact of PGE_2 depletion on tissue healing in mind (79-81), its impact on the tissue repair and resolution needs to be thoroughly investigated.

Most importantly, apart from providing new insights on PGE_2, numerous crucial mechanistic and therapeutic questions remain unanswered. To begin with, the source of PGE_2 in mediating the peripheral effects of CAP is unclear and can be discovered by carrying out adoptive bone marrow transfer experiments using mPGES-1 gene deleted mice. Furthermore, the effects of VNS on neuroinflammatory cytokines and involvement of mPGES-1 dependent PGE_2 needs to be elucidated. The expression pattern of EP receptors and their role in PGE_2 mediated immunosuppression is yet to be addressed. Another critical question is the impact of mPGES-1 gene deletion on the generation of SPMs and this can be addressed by measuring their levels in mouse samples following VNS. Interestingly, given the therapeutic effects of VNS in patients with chronic inflammation (64, 168), the effects of mPGES-1 depletion on chronic inflammatory mice models such as arthritis undergoing VNS needs to be explored. Epidemiological studies on the effects of NSAIDS in patients receiving VNS

therapy might further expand our knowledge on the anti-cholinergic effects of NSAIDS on inflammation. In addition, studies of NSAIDs and mPGES-1 inhibitor effects in this context may expand our current knowledge on the exact mechanisms of action of these drugs, as well as confer new data on their specific interaction with cholinergic immune regulation.

In conclusion, both our *in vitro* and *in vivo* data show that PGE_2 has a crucial role in cholinergic immune regulatory mechanisms.

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